



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C07H 21/02, 21/04, C07K 1/00, 14/00, C12N 5/00, 15/09, 15/63	A1	(11) International Publication Number: WO 97/33902 (43) International Publication Date: 18 September 1997 (18.09.97)
(21) International Application Number: PCT/US96/03774 (22) International Filing Date: 14 March 1996 (14.03.96) (71) Applicant (for all designated States except US): HUMAN GENOME SCIENCES, INC. [US/US]; 9410 Key West Avenue, Rockville, MD 20850-3338 (US). (72) Inventors; and (75) Inventors/Applicants (for US only): NI, Jian [CN/US]; 5502 Manorfield Road, Rockville, MD 20853 (US). YU, Guo-Liang [CN/US]; 13524 Straw Bale Lane, Darnestown, MD 20878 (US). GENTZ, Reiner, L. [DE/US]; 13404 Fairland Park Drive, Silver Spring, MD 20904 (US). (74) Agents: HERRON, Charles, J.; Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart & Olstein, 6 Becker Farm Road, Roseland, NJ 07068 (US) et al.		(81) Designated States: AM, AU, BG, BR, BY, CA, CN, CZ, EE, FI, GE, HU, JP, KG, KP, KR, KZ, LT, LV, MD, MN, MX, NO, NZ, PL, RO, RU, SG, SI, SK, TJ, TM, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>
(54) Title: HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON (57) Abstract The invention relates to human TNF delta and TNF epsilon polypeptides, polynucleotides encoding the polypeptides, methods for producing the polypeptides, in particular by expressing the polynucleotides, and agonists and antagonists of the polypeptides. The invention further relates to methods for utilizing such polynucleotides, polypeptides, agonists and antagonists for applications, which relate, in part, to research, diagnostic and clinical arts.		

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Date
15.09.04

Reference
C 2276 EP

Application No./Patent No.
96910483.5 - 2101 / 0897390

Applicant/Proprietor
HUMAN GENOME SCIENCES, INC.

Communication regarding the expiry of the time limit within which notice of opposition may be filed

You are hereby informed that on expiry of the nine-month time limit from the publication of the mention of the grant of European Patent No 0897390 no notice of opposition had reached the files.

The entry in the Register of European Patents will be automatically generated by the electronic data processing system.

Examining Division



EPPU 02: 12.11.03 2101



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Datum/Date

02/10/03

Zeichen/Ref./Réf. C 2276 EP	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°. 96910483.5-2109 0897390
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire HUMAN GENOME SCIENCES, INC.	

DECISION TO GRANT A EUROPEAN PATENT PURSUANT TO ARTICLE 97(2) EPC

Following examination of European patent application No. 96910483.5 a European patent with the title and the supporting documents indicated in the communication pursuant to Rule 51(4) EPC dated 17.02.03 is hereby granted in respect of the designated Contracting States.

The modifications subsequently requested by the applicant and received at the EPO on 27.06.03 have been taken into account.

Patent No. : 0897390
Date of filing : 14.03.96
Priority claimed :
Designated Contracting States
and Proprietor(s) : AT-BE-CH-DE-DK-ES-FI-FR-GB-GR-IE-IT-LI-
LU-MC-NL-PT-SE
HUMAN GENOME SCIENCES, INC.
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This decision will take effect on the date on which the European Patent Bulletin mentions the grant (Art. 97(4) and (5) EPC).

The mention of the grant will be published in European Patent Bulletin 03/46 of 12.11.03.

Examining Division
BARDILI W B

HENNARD C

DEFFNER C A E



Registered letter

**ANMERKUNG ZUR ENTSCHEIDUNG ÜBER DIE ERTEILUNG
EINES EUROPÄISCHEN PATENTS (EPA Form 2006)**

1. **EPA Informationsbroschüre "Nationales Recht zum EPÜ"**
Diese Broschüre enthält nützliche Informationen zu den formalen Erfordernissen und den Handlungen, die vor den Patentbehörden der Vertragsstaaten vorzunehmen sind, um Rechte in diesen Staaten zu erlangen. Da diese Handlungen einem ständigen Wandel unterworfen sind, sollte immer nur die neueste Ausgabe der Broschüre benutzt werden. Nächträgliche Informationen werden im Amtsblatt veröffentlicht.
2. **Übersetzung der europäischen Patentschrift nach Artikel 65(1) des Europäischen Patentübereinkommens**
Sie werden erneut darauf hingewiesen, dass bestimmte Vertragsstaaten nach Artikel 65(1) EPÜ eine Übersetzung der europäischen Patentschrift verlangen; hierauf wird in der Mitteilung gemäss Regel 51(6) verwiesen. Die Nichteinreichung dieser Übersetzung kann zur Folge haben, dass das Patent in dem betreffenden Staat/in den betreffenden Staaten als von Anfang an nicht eingetreten gilt. Weitere Einzelheiten entnehmen Sie bitte der oben genannten Broschüre.
3. **Zahlung von Jahresgebühren für europäische Patente**
Nach Artikel 141 EPU können "nationale" Jahresgebühren für das europäische Patent für die Jahre erhoben werden, die an das Jahr anschliessen, in dem der Hinweis auf die Erteilung des europäischen Patents im "Europäischen Patentblatt" bekanntgemacht wird. Weitere Einzelheiten entnehmen Sie bitte der oben genannten Broschüre.

**NOTE RELATING TO THE DECISION TO GRANT A
EUROPEAN PATENT (EPO Form 2006)**

1. **EPO Information Brochure "National law relating to the EPC"**
This brochure provides useful information regarding formal requirements and the steps to be taken before the patent authorities of the Contracting States in order to acquire rights in those states. Since the necessary steps are subject to change the latest edition of the brochure should always be used. Subsequent information is published in the Official Journal.
2. **Translation of the European patent specification under Article 65(1) of the European Patent Convention**
Your attention is again drawn to the requirements regarding translation of the European patent specification laid down by a number of Contracting States under Article 65(1) EPC, to which reference is made in the communication under Rule 51(6). Failure to supply such translation(s) may result in the patent being deemed to be void "ab initio" in the State(s) in question. For further details you are recommended to consult the above-mentioned brochure.
3. **Payment of renewal fees for European patents**
Under Article 141 EPC "national" renewal fees in respect of a European patent may be imposed for the years which follow that in which the mention of the grant of the European patent is published in the "European Patent Bulletin". For further details you are recommended to consult the above-mentioned brochure.

**REMARQUE RELATIVE A LA DECISION DE DELIVRANCE
D'UN BREVET EUROPEEN (OEB Form 2006)**

1. **Brochure d'information de l'OEB "Droit national relatif à la CBE"**
Cette brochure fournit d'utiles renseignements sur les conditions de forme requises et sur les actes à accomplir auprès des offices de brevet des Etats contractants aux fins d'obtenir des droits dans les Etats contractants. Etant donné que les actes indispensables sont susceptibles de modifications, il serait bon de toujours consulter la dernière édition de la brochure. Toute information ultérieure est publiée au Journal Officiel.
2. **Traduction du fascicule du brevet européen en vertu de l'article 65(1) de la Convention sur le brevet européen**
Votre attention est de nouveau attirée sur l'obligation faite par certains Etats contractants, en vertu de l'article 65(1) CBE, de fournir une traduction du fascicule du brevet européen, à laquelle il est fait référence dans la notification établie conformément à la règle 51(6). Si la(les) traduction(s) n'est(ne sont) pas fournie(s), le brevet européen peut, dès l'origine, être réputé sans effet dans cet(ces) Etat(s). Pour plus de détails, nous vous renvoyons à la brochure susmentionnée.
3. **Paiement des taxes annuelles pour le brevet européen**
Conformément à l'article 141 CBE, les taxes annuelles "nationales" dues au titre du brevet européen peuvent être perçues pour les années suivant celle au cours de laquelle la mention de la délivrance du brevet européen est publiée au "Bulletin européen des brevets". Pour plus de détails, nous vous renvoyons à la brochure susmentionnée.

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Partnerschaftsregister Amtsgericht München PR 89

EP 96 91 0483.5-2109
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

EPO - Munich
66
13. Aug. 2003

August 11, 2003
JAE/OOV

This is in response to your BRIEF COMMUNICATION dated August 7, 2003.

Enclosed please find the confirmation of the cited documents.



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosure:
confirmation

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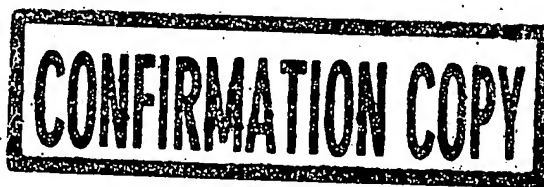
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EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

8. Juli 2003

~~June 26, 2003~~

JAE/OOV



This is in reply to the Rule 51(4) Communication issued on February 17, 2003:

1. We request the correction of the following grammatical and typographical errors on page 1 of the claims set:

claim 1(f), last line: delete "and" after "...response,";

claim 1(g), last line: replace "." with ","; and

claim 1(h), last line: insert "and" after "...response,".

We enclose correspondingly amended page 1 of the claims set.

2. Applicants declare their approval of the text as specified in the Rule 51(4) Communication provided that the amendments requested above will be accepted.
3. Furthermore, we herewith enclose the German and French translations of the claims as allowed.

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4. It is requested that 3 copies of the printed patent specification be supplied together with the Certificate for a European Patent.

5. Fees payable to the EPO:

- Fee for grant and printing (64 pages)	EUR 1.145,00
- Fee for 3 printed patent specifications	EUR 12,00
Total	<u>EUR 1.157,00</u>

The deduction of the total amount in EUR is to be made upon receipt of the enclosed debit order from our deposit account No. 2800.0321 at the EPO in Munich.



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosures:

Amended page 1 of the claims set
German claims; in dupl.
French claims; in dupl.
Order to debit account

PATENTANSPRÜCHE

1. Polynucleotid, ausgewählt aus der Gruppe bestehend aus
 - (a) Polynucleotiden, die mindestens die reife Form des Polypeptids codieren, das die abgeleitete Aminosäuresequenz wie in Figur 1 oder 2 dargestellt hat;
 - (b) Polynucleotiden mit der codierenden Sequenz wie in Figur 1 oder 2 dargestellt, die mindestens die reife Form des Polypeptids codieren;
 - (c) Polynucleotiden, die das Polypeptid mit der Aminosäuresequenz mindestens der reifen Form des Polypeptids codieren, das von der cDNA, die in ATCC 97377 oder ATCC 97457 enthalten ist, codiert wird;
 - (d) Polynucleotiden mit der codierenden Sequenz der cDNA, die in ATCC 97377 oder ATCC 97457 enthalten ist, die mindestens die reife Form des Polypeptids codieren;
 - (e) Polynucleotiden, die eine Aminosäuresequenz codieren, die von einem Polynucleotid aus (a) bis (d) codiert wird, wobei 1 bis 5 oder 5 bis 10 Aminosäuren in beliebiger Kombination substituiert, entfernt oder hinzugefügt werden;
 - (f) Polynucleotiden, die ein Polypeptid codieren, das ein Fragment eines Polypeptids mit einer Länge von mindestens 30 oder mindestens 50 Aminosäuren umfasst, das von einem Polynucleotid aus (a) bis (d) codiert wird, wobei das Fragment in der Lage ist, eine Immunantwort zu stimulieren;
 - (g) Polynucleotiden wie in (f) definiert, die funktionell mit einer heterologen regulatorischen Sequenz verbunden sind;
 - (h) Polynucleotiden, die mindestens zu 70% mit einem Polynucleotid aus (a) bis (d) identisch sind und die ein Polypeptid codieren, das in der Lage ist, eine Immunantwort zu stimulieren; und
 - (i) Polynucleotiden, die ein Polypeptid codieren, das mindestens zu 70%

mit einem Polypeptid identisch ist, das von einem Polynucleotid aus (a) bis (d) codiert wird;

oder der komplementäre Strang eines solchen Polynucleotids.

2. Polynucleotid nach Anspruch 1, das eine DNA oder RNA ist.
3. DNA nach Anspruch 2, die eine genomische DNA ist.
4. Polynucleotid nach einem der Ansprüche 1 bis 3, das mit einem heterologen Polynucleotid fusioniert ist.
5. Vektor, der das Polynucleotid nach einem der Ansprüche 1 bis 4 enthält.
6. Vektor nach Anspruch 5, wobei das Polynucleotid mit Expressionskontrollsequenzen funktionell verbunden ist, die die Expression in prokaryontischen oder eukaryontischen Wirtszellen gestatten.
7. Wirtszelle, die mit dem Polynucleotid nach einem der Ansprüche 1 bis 4 oder dem Vektor nach Anspruch 5 oder 6 genetisch verändert wurde.
8. Verfahren zur Herstellung eines Polypeptids, das in der Lage ist, eine Immunantwort zu stimulieren, wobei das Verfahren die folgenden Schritte umfasst: Züchten der Wirtszelle nach Anspruch 7 und Gewinnen des von dem Polynucleotid codierten Polypeptids aus der Kultur.
9. Verfahren zur Herstellung von Zellen, die in der Lage sind, ein Polypeptid zu exprimieren, das in der Lage ist, eine Immunantwort zu stimulieren, wobei das Verfahren die genetische Veränderung von Zellen mit dem Vektor nach Anspruch 5 oder 6 umfasst.
10. Polypeptid mit der Aminosäuresequenz, die von einem Polynucleotid nach einem der Ansprüche 1 bis 4 codiert wird, oder das durch das Verfahren nach Anspruch 8 erhältlich ist.

11. Antikörper, der spezifisch für das Polypeptid nach Anspruch 10 ist.
12. Nucleinsäuremolekül, das spezifisch mit einem Polynucleotid nach einem der Ansprüche 1 bis 4 hybridisiert.
13. Antagonist/Inhibitor des Polypeptids nach Anspruch 10, wobei der Antagonist/Inhibitor ein Antikörper nach Anspruch 11 ist und in der Lage ist, die Aktivität des Polypeptids nach Anspruch 10 zu hemmen oder auszuschalten, oder ein Nucleinsäuremolekül nach Anspruch 12, das in der Lage ist, das Polynucleotid oder die DNA nach einem der Ansprüche 1 bis 4 zu binden und dadurch dessen Expression zu hemmen.
14. Arzneimittel, umfassend das Polynucleotid nach einem der Ansprüche 1 bis 4, das Polypeptid nach Anspruch 10 oder eine DNA, die das Polypeptid codiert und in der Lage ist, es in vivo zu exprimieren, oder den Antagonist/Inhibitor nach Anspruch 13 und gegebenenfalls einen pharmazeutisch verträglichen Träger.
15. Diagnostische Zusammensetzung, umfassend das Polynucleotid nach einem der Ansprüche 1 bis 4, das Nucleinsäuremolekül nach Anspruch 12 oder den Antikörper nach Anspruch 11.
16. Verwendung des Polypeptids nach Anspruch 10 oder des Polynucleotids nach einem der Ansprüche 1 bis 4 für die Herstellung eines Arzneimittels zur Behandlung von Neoplasie, zur Wundheilung, zur Behandlung von Restenose, zur Regulierung der Hämatopoiese in der Entwicklung von Endothelzellen, zur Stimulierung einer Immunantwort gegen parasitäre, bakterielle oder virale Infektionen, oder zur Behandlung und/oder Vorbeugung von Autoimmunerkrankungen.
17. Verwendung des Antagonisten/Inhibitors nach Anspruch 13 für die Herstellung eines Arzneimittels zur Behandlung von Cachexie, Gehirnmalaria,

rheumatoider Arthritis, zur Vorbeugung der Transplantat-Wirt-Abstoßung, zur Hemmung von Knochenresorption, zur Behandlung und/oder Vorbeugung von Osteoporose, oder zur Behandlung von endotoxischem Schock.

18. Verfahren zur Diagnose einer Krankheit oder der Neigung zu einer Krankheit, die mit einer Unterexpression des Polypeptids nach Anspruch 10 in Zusammenhang steht, umfassend die Bestimmung einer Mutation in einer Nucleinsäuresequenz, die das Polypeptid codiert.
19. Diagnostisches Verfahren, umfassend das Analysieren auf das Vorliegen eines Polypeptids nach Anspruch 10 in einer Probe, die einem Wirt entnommen wurde.
20. Verfahren zur Identifizierung von Verbindungen, die das Polypeptid nach Anspruch 10 binden und seine Aktivierung hemmen, umfassend
 - (a) Inkontaktbringen einer Zelle, die an ihrer Oberfläche einen Rezeptor für das Polypeptid exprimiert, wobei der Rezeptor mit einem zweiten Bestandteil assoziiert ist, der in der Lage ist, nach Binden einer Verbindung an den Rezeptor ein nachweisbares Signal zu liefern, mit einem analytisch nachweisbaren TNF-delta-Polypeptid und einer Verbindung unter Bedingungen, die eine Bindung an den Rezeptor gestatten; und
 - (b) Feststellen, ob die Verbindung an den Rezeptor bindet und diesen hemmt, indem die Abwesenheit eines Signals nachgewiesen wird, das durch die Wechselwirkung von TNF-delta mit dem Rezeptor erzeugt wird.

REVENDICATIONS

1- Polynucléotide sélectionné parmi le groupe constitué de

- (a) polynucléotides codant pour au moins la forme mature du polypeptide présentant la séquence d'acide aminé déduite décrite dans la Figure 1 ou 2 ;
- (b) polynucléotides présentant la séquence codante décrite dans la Figure 1 ou 2 codant pour au moins la forme mature du polypeptide ;
- (c) polynucléotides codant pour le polypeptide présentant la séquence d'acide aminé d'au moins la forme mature du polypeptide codé par l'ADNc contenu dans ATCC 97377 ou ATCC 97457 ;
- (d) polynucléotides présentant la séquence codante de l'ADNc contenu dans ATCC 97377 ou ATCC 97457 codant pour au moins la forme mature du polypeptide ;
- (e) polynucléotides codant pour une séquence d'acide aminé codée par l'un quelconque des polynucléotides selon (a) à (d), dans laquelle 1 à 5 ou 5 à 10 acides aminés sont substitués, supprimés ou ajoutés, selon une combinaison quelconque ;
- (f) polynucléotides codant pour un polypeptide comprenant un fragment d'une longueur d'au moins 30 ou au moins 50 acides aminés d'un polypeptide codé par l'un quelconque des polynucléotides selon (a) à (d), ledit fragment étant capable de stimuler une réponse immunitaire ;
- (g) polynucléotides tels que définis en (f) qui sont liés de manière opérationnelle à une séquence régulatrice hétérologue ;
- (h) polynucléotides qui ont au moins 70 % d'identité avec l'un quelconque des polynucléotides selon (a) à (d) et qui codent pour un polypeptide capable de stimuler une réponse immunitaire ; et
- (i) polynucléotides codant pour un polypeptide qui a au moins 70 % d'identité avec un polypeptide codé par l'un quelconque des polynucléotides selon (a) à (d) ;

ou le brin complémentaire d'un tel polynucléotide.

2- Polynucléotide selon la revendication 1 qui est un ADN ou un ARN.

3- ADN selon la revendication 2 qui est de l'ADN génomique.

4- Polynucléotide selon l'une des revendications 1 à 3 qui est fusionné à un polynucléotide hétérologue.

5- Vecteur contenant un polynucléotide selon l'une des revendications 1 à 4.

6- Vecteur selon la revendication 5 dans lequel le polynucléotide est lié de manière opérationnelle à des séquences de contrôle d'expression permettant l'expression dans des cellules hôtes procaryotes ou eucaryotes.

7- Cellule hôte génétiquement modifiée avec un polynucléotide selon l'une des revendications 1 à 4 ou un vecteur selon la revendication 5 ou 6.

8- Procédé de production d'un polypeptide capable de stimuler une réponse immunitaire comprenant : cultiver la cellule hôte selon la revendication 7 et récupérer le polypeptide codé par ledit polynucléotide à partir de la culture.

9- Procédé de production de cellules capables d'exprimer un polypeptide qui est capable de stimuler une réponse immunitaire comprenant la modification génétique de cellules avec un vecteur selon la revendication 5 ou 6.

10- Polypeptide présentant une séquence d'acide aminé codée par un polynucléotide selon l'une des revendications 1 à 4 ou susceptible d'être obtenu par le procédé selon la revendication 8.

11- Anticorps spécifique d'un polypeptide selon la revendication 10.

12- Molécule d'acide nucléique qui s'hybride spécifiquement à un polynucléotide selon l'une des revendications 1 à 4.

13- Antagoniste/inhibiteur d'un polypeptide selon la revendication 10, dans lequel ledit antagoniste/inhibiteur est un anticorps selon la revendication 11 capable d'inhiber ou de supprimer l'activité du polypeptide selon la revendication 10 ou une molécule d'acide nucléique selon la revendication 12 capable de lier et par cela d'inhiber l'expression d'un polynucléotide ou ADN selon l'une des revendications 1 à 4.

14- Composition pharmaceutique comprenant le polynucléotide selon l'une des revendications 1 à 4, le polypeptide selon la revendication 10 ou un ADN codant et capable d'exprimer ledit polypeptide in vivo ou l'antagoniste/inhibiteur selon la revendication 13 et facultativement un véhicule pharmaceutiquement acceptable.

15- Composition diagnostique comprenant le polynucléotide selon l'une des revendications 1 à 4, la molécule d'acide nucléique selon la revendication 12 ou l'anticorps selon la revendication 11.

16- Utilisation du polypeptide selon la revendication 10 ou du polynucléotide selon l'une des revendications 1 à 4 pour la préparation d'une composition pharmaceutique destinée au traitement de la néoplasie, pour la cicatrisation, pour le traitement de la resténose, pour réguler l'hématopoïèse dans le développement de cellule endothéliale, pour stimuler une réponse immunitaire contre des infections parasitaires, bactériennes ou virales, ou pour le traitement et/ou la prévention de maladies autoimmunes.

17- Utilisation de l'antagoniste/inhibiteur selon la revendication 13 pour la préparation d'une composition pharmaceutique destinée au traitement de la cachexie, d'un accès pernicieux, de l'arthrite rhumatoïde, pour la prévention de rejet de greffe, pour inhiber la résorption osseuse, pour le traitement et/ou la prévention de l'ostéoporose, ou pour le traitement du choc endotoxique.

18- Procédé de diagnostic d'une maladie ou d'une susceptibilité à une maladie liée à une sous-expression du polypeptide selon la revendication 10 comprenant la détermination d'une mutation dans une séquence d'acide nucléique codant pour ledit polypeptide.

19- Procédé de diagnostic comprenant l'analyse de la présence du polypeptide selon la revendication 10 dans un échantillon dérivé d'un hôte.

20- Méthode d'identification de composés qui se lient et inhibent l'activation du polypeptide selon la revendication 10 comprenant :

- (a) mettre en contact une cellule exprimant à sa surface un récepteur au polypeptide, ledit récepteur étant associé à un second élément capable de produire un signal détectable en réponse à la liaison d'un composé audit récepteur, avec un polypeptide TNF delta analytiquement détectable et un composé dans des conditions permettant la liaison au récepteur ; et
- (b) déterminer si le composé se lie et inhibe le récepteur par la détection de l'absence d'un signal généré par l'interaction du TNF delta avec le récepteur.

EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

Druckexemplar

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polypeptide encoded by a polynucleotide of any one of (a) to (d) wherein said fragment is capable of stimulating an immune response; ~~and~~ X
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence; X
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response; *and* X
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.

2. The polynucleotide of claim 1 which is DNA or RNA.

3. The DNA of claim 2 which is genomic DNA.



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Datum/Date 07-08-2003

Zeichen/Ref./Réf. C 2276 EP	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°. 96910483.5-2109/0897390
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire HUMAN GENOME SCIENCES, INC.	

BRIEF COMMUNICATION

Subject: ☐ Your letter of
☐ Our telephone conversation of
☐ Communication of
☐

Enclosure(s): ☐ Letter from the proprietor of the patent/opponent of

☐ Copy(copies)

☐ Communication:

with regard to your Fax dated 08-07-03 concerning a correction request,
we have to inform you that the confirmation of the cited documents is
required(Claimstran in FRENCH AND GERMAN).

Please ,send the confirmation a.s.a.p. in order to get ready your publication.

We apologise for any inconvenience that this situation might cause you.

Please take note.

Ruiz, José
Formalities Officer
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☐ REGISTERED LETTER



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Datum/Date

29/07/03

Zeichen/Ref./Réf. C 2276 EP	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°. 96910483.5-2109 0897390
Anmelder/Applicant/Demandeur/Patentinhaber/Propriétaire/Titulaire HUMAN GENOME SCIENCES, INC.	

Brief communication

Subject: Request for amendment of the application documents

dated ..08.07.03.....

Your request for amendments under Rule 86(3) EPC/corrections under Rule 88 EPC was allowed.

☒ the replacement pages have been used

☐ a copy of the requested amendments made is attached hereto

For the Examining Division:
WERNER H M
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i.v. Antonio Gallego-8102



Application No.:

96 910 483.5

IV.1 Documents for the grant of a patent

Text for the Contracting States:

AT BE CH LI DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Description, pages:

1,3-7,9-63	as published		
2,8,64	as received on	19.12.2002 with letter of	18.12.2002

Claims, No.:

4-20	as received on	19.12.2002 with letter of	18.12.2002
1-3	as received on	27.06.2003 with letter of	26.06.2003

Drawings, sheets:

1/10,3/10-10/10	as published		
2/10	as received on	18.02.2002 with letter of	14.02.2002

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Partnerschaftsregister Amtsgericht München PR 89

EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

8. Juli 2003

~~June 26, 2003~~

JAE/OOV

According to our recent telephone conversation, enclosed please find a copy of the French and German claims set, which was already filed with petition dated June 26, 2003. Vossius & Partner

This is in reply to the Rule 51(4) Communication issued on February 17, 2003:

1. We request the correction of the following grammatical and typographical errors on page 1 of the claims set:

claim 1(f), last line: delete "and" after "...response,";

claim 1(g), last line: replace "." with ","; and

claim 1(h), last line: insert "and" after "...response,".

Zur Kasse

€ 1157,- (A)

We enclose correspondingly amended page 1 of the claims set.

2. Applicants declare their approval of the text as specified in the Rule 51(4) Communication provided that the amendments requested above will be accepted.
3. Furthermore, we herewith enclose the German and French translations of the claims as allowed.

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Empfangszeit 8. Juli 10:23

4. It is requested that 3 copies of the printed patent specification be supplied together with the Certificate for a European Patent.

5. Fees payable to the EPO:

- Fee for grant and printing (64 pages)	EUR 1.145,00
- Fee for 3 printed patent specifications	EUR 12,00
Total	<u>EUR 1.157,00</u>

The deduction of the total amount in EUR is to be made upon receipt of the enclosed debit order from our deposit account No. 2800.0321 at the EPO in Munich.



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosures:

Amended page 1 of the claims set
German claims, in dupl.
French claims, in dupl.
Order to debit account

PATENTANSPRÜCHE

1. Polynucleotid; ausgewählt aus der Gruppe bestehend aus
 - (a) Polynucleotiden, die mindestens die reife Form des Polypeptids codieren, das die abgeleitete Aminosäuresequenz wie in Figur 1 oder 2 dargestellt hat;
 - (b) Polynucleotiden mit der codierenden Sequenz wie in Figur 1 oder 2 dargestellt, die mindestens die reife Form des Polypeptids codieren;
 - (c) Polynucleotiden, die das Polypeptid mit der Aminosäuresequenz mindestens der reifen Form des Polypeptids codieren, das von der cDNA, die in ATCC 97377 oder ATCC 97457 enthalten ist, codiert wird;
 - (d) Polynucleotiden mit der codierenden Sequenz der cDNA, die in ATCC 97377 oder ATCC 97457 enthalten ist, die mindestens die reife Form des Polypeptids codieren;
 - (e) Polynucleotiden, die eine Aminosäuresequenz codieren, die von einem Polynucleotid aus (a) bis (d) codiert wird, wobei 1 bis 5 oder 5 bis 10 Aminosäuren in beliebiger Kombination substituiert, entfernt oder hinzugefügt werden;
 - (f) Polynucleotiden, die ein Polypeptid codieren, das ein Fragment eines Polypeptids mit einer Länge von mindestens 30 oder mindestens 50 Aminosäuren umfasst, das von einem Polynucleotid aus (a) bis (d) codiert wird, wobei das Fragment in der Lage ist, eine Immunantwort zu stimulieren;
 - (g) Polynucleotiden wie in (f) definiert, die funktionell mit einer heterologen regulatorischen Sequenz verbunden sind;
 - (h) Polynucleotiden, die mindestens zu 70% mit einem Polynucleotid aus (a) bis (d) identisch sind und die ein Polypeptid codieren, das in der Lage ist, eine Immunantwort zu stimulieren; und
 - (i) Polynucleotiden, die ein Polypeptid codieren, das mindestens zu 70%

mit einem Polypeptid identisch ist, das von einem Polynucleotid aus (a) bis (d) codiert wird;
oder der komplementäre Strang eines solchen Polynucleotids.

2. Polynucleotid nach Anspruch 1, das eine DNA oder RNA ist.
3. DNA nach Anspruch 2, die eine genomische DNA ist.
4. Polynucleotid nach einem der Ansprüche 1 bis 3, das mit einem heterologen Polynucleotid fusioniert ist.
5. Vektor, der das Polynucleotid nach einem der Ansprüche 1 bis 4 enthält.
6. Vektor nach Anspruch 5, wobei das Polynucleotid mit Expressionskontrollsequenzen funktionell verbunden ist, die die Expression in prokaryontischen oder eukaryontischen Wirtszellen gestatten.
7. Wirtszelle, die mit dem Polynucleotid nach einem der Ansprüche 1 bis 4 oder dem Vektor nach Anspruch 5 oder 6 genetisch verändert wurde.
8. Verfahren zur Herstellung eines Polypeptids, das in der Lage ist, eine Immunantwort zu stimulieren, wobei das Verfahren die folgenden Schritte umfasst: Züchten der Wirtszelle nach Anspruch 7 und Gewinnen des von dem Polynucleotid codierten Polypeptids aus der Kultur.
9. Verfahren zur Herstellung von Zellen, die in der Lage sind, ein Polypeptid zu exprimieren, das in der Lage ist, eine Immunantwort zu stimulieren, wobei das Verfahren die genetische Veränderung von Zellen mit dem Vektor nach Anspruch 5 oder 6 umfasst.
10. Polypeptid mit der Aminosäuresequenz, die von einem Polynucleotid nach einem der Ansprüche 1 bis 4 codiert wird, oder das durch das Verfahren nach Anspruch 8 erhältlich ist.

11. Antikörper, der spezifisch für das Polypeptid nach Anspruch 10 ist.
12. Nucleinsäuremolekül, das spezifisch mit einem Polynucleotid nach einem der Ansprüche 1 bis 4 hybridisiert.
13. Antagonist/Inhibitor des Polypeptids nach Anspruch 10, wobei der Antagonist/Inhibitor ein Antikörper nach Anspruch 11 ist und in der Lage ist, die Aktivität des Polypeptids nach Anspruch 10 zu hemmen oder auszuschalten, oder ein Nucleinsäuremolekül nach Anspruch 12, das in der Lage ist, das Polynucleotid oder die DNA nach einem der Ansprüche 1 bis 4 zu binden und dadurch dessen Expression zu hemmen.
14. Arzneimittel, umfassend das Polynucleotid nach einem der Ansprüche 1 bis 4, das Polypeptid nach Anspruch 10 oder eine DNA, die das Polypeptid codiert und in der Lage ist, es in vivo zu exprimieren, oder den Antagonist/Inhibitor nach Anspruch 13 und gegebenenfalls einen pharmazeutisch verträglichen Träger.
15. Diagnostische Zusammensetzung, umfassend das Polynucleotid nach einem der Ansprüche 1 bis 4, das Nucleinsäuremolekül nach Anspruch 12 oder den Antikörper nach Anspruch 11.
16. Verwendung des Polypeptids nach Anspruch 10 oder des Polynucleotids nach einem der Ansprüche 1 bis 4 für die Herstellung eines Arzneimittels zur Behandlung von Neoplasie, zur Wundheilung, zur Behandlung von Restenose, zur Regulierung der Hämatopoiese in der Entwicklung von Endothelzellen, zur Stimulierung einer Immunantwort gegen parasitäre, bakterielle oder virale Infektionen, oder zur Behandlung und/oder Vorbeugung von Autoimmunerkrankungen.
17. Verwendung des Antagonisten/Inhibitors nach Anspruch 13 für die Herstellung eines Arzneimittels zur Behandlung von Cachexie, Gehimmlaria,

rheumatoider Arthritis, zur Vorbeugung der Transplantat-Wirt-Abstoßung, zur Hemmung von Knochenresorption, zur Behandlung und/oder Vorbeugung von Osteoporose, oder zur Behandlung von endotoxischem Schock.

18. Verfahren zur Diagnose einer Krankheit oder der Neigung zu einer Krankheit, die mit einer Unterexpression des Polypeptids nach Anspruch 10 in Zusammenhang steht, umfassend die Bestimmung einer Mutation in einer Nucleinsäuresequenz, die das Polypeptid codiert.
19. Diagnostisches Verfahren, umfassend das Analysieren auf das Vorliegen eines Polypeptids nach Anspruch 10 in einer Probe, die einem Wirt entnommen wurde.
20. Verfahren zur Identifizierung von Verbindungen, die das Polypeptid nach Anspruch 10 binden und seine Aktivierung hemmen, umfassend
 - (a) Inkontaktbringen einer Zelle, die an ihrer Oberfläche einen Rezeptor für das Polypeptid exprimiert, wobei der Rezeptor mit einem zweiten Bestandteil assoziiert ist, der in der Lage ist, nach Binden einer Verbindung an den Rezeptor ein nachweisbares Signal zu liefern, mit einem analytisch nachweisbaren TNF-delta-Polypeptid und einer Verbindung unter Bedingungen, die eine Bindung an den Rezeptor gestatten; und
 - (b) Feststellen, ob die Verbindung an den Rezeptor bindet und diesen hemmt, indem die Abwesenheit eines Signals nachgewiesen wird, das durch die Wechselwirkung von TNF-delta mit dem Rezeptor erzeugt wird.

REVENDECATIONS

1- Polynucléotide sélectionné parmi le groupe constitué de

- (a) polynucléotides codant pour au moins la forme mature du polypeptide présentant la séquence d'acide aminé déduite décrite dans la Figure 1 ou 2 ;
- (b) polynucléotides présentant la séquence codante décrite dans la Figure 1 ou 2 codant pour au moins la forme mature du polypeptide ;
- (c) polynucléotides codant pour le polypeptide présentant la séquence d'acide aminé d'au moins la forme mature du polypeptide codé par l'ADNc contenu dans ATCC 97377 ou ATCC 97457 ;
- (d) polynucléotides présentant la séquence codante de l'ADNc contenu dans ATCC 97377 ou ATCC 97457 codant pour au moins la forme mature du polypeptide ;
- (e) polynucléotides codant pour une séquence d'acide aminé codée par l'un quelconque des polynucléotides selon (a) à (d), dans laquelle 1 à 5 ou 5 à 10 acides aminés sont substitués, supprimés ou ajoutés, selon une combinaison quelconque ;
- (f) polynucléotides codant pour un polypeptide comprenant un fragment d'une longueur d'au moins 30 ou au moins 50 acides aminés d'un polypeptide codé par l'un quelconque des polynucléotides selon (a) à (d), ledit fragment étant capable de stimuler une réponse immunitaire ;
- (g) polynucléotides tels que définis en (f) qui sont liés de manière opérationnelle à une séquence régulatrice hétérologue ;
- (h) polynucléotides qui ont au moins 70 % d'identité avec l'un quelconque des polynucléotides selon (a) à (d) et qui codent pour un polypeptide capable de stimuler une réponse immunitaire ; et
- (i) polynucléotides codant pour un polypeptide qui a au moins 70 % d'identité avec un polypeptide codé par l'un quelconque des polynucléotides selon (a) à (d) ;

ou le brin complémentaire d'un tel polynucléotide.

2- Polynucléotide selon la revendication 1 qui est un ADN ou un ARN.

3- ADN selon la revendication 2 qui est de l'ADN génomique.

4- Polynucléotide selon l'une des revendications 1 à 3 qui est fusionné à un polynucléotide hétérologue.

5- Vecteur contenant un polynucléotide selon l'une des revendications 1 à 4.

6- Vecteur selon la revendication 5 dans lequel le polynucléotide est lié de manière opérationnelle à des séquences de contrôle d'expression permettant l'expression dans des cellules hôtes procaryotes ou eucaryotes.

7- Cellule hôte génétiquement modifiée avec un polynucléotide selon l'une des revendications 1 à 4 ou un vecteur selon la revendication 5 ou 6.

8- Procédé de production d'un polypeptide capable de stimuler une réponse immunitaire comprenant : cultiver la cellule hôte selon la revendication 7 et récupérer le polypeptide codé par ledit polynucléotide à partir de la culture.

9- Procédé de production de cellules capables d'exprimer un polypeptide qui est capable de stimuler une réponse immunitaire comprenant la modification génétique de cellules avec un vecteur selon la revendication 5 ou 6.

10- Polypeptide présentant une séquence d'acide aminé codée par un polynucléotide selon l'une des revendications 1 à 4 ou susceptible d'être obtenu par le procédé selon la revendication 8.

11- Anticorps spécifique d'un polypeptide selon la revendication 10.

12- Molécule d'acide nucléique qui s'hybride spécifiquement à un polynucléotide selon l'une des revendications 1 à 4.

13- Antagoniste/inhibiteur d'un polypeptide selon la revendication 10, dans lequel ledit antagoniste/inhibiteur est un anticorps selon la revendication 11 capable d'inhiber ou de supprimer l'activité du polypeptide selon la revendication 10 ou une molécule d'acide nucléique selon la revendication 12 capable de lier et par cela d'inhiber l'expression d'un polynucléotide ou ADN selon l'une des revendications 1 à 4.

14- Composition pharmaceutique comprenant le polynucléotide selon l'une des revendications 1 à 4, le polypeptide selon la revendication 10 ou un ADN codant et capable d'exprimer ledit polypeptide in vivo ou l'antagoniste/inhibiteur selon la revendication 13 et facultativement un véhicule pharmaceutiquement acceptable.

15- Composition diagnostique comprenant le polynucléotide selon l'une des revendications 1 à 4, la molécule d'acide nucléique selon la revendication 12 ou l'anticorps selon la revendication 11.

16- Utilisation du polypeptide selon la revendication 10 ou du polynucléotide selon l'une des revendications 1 à 4 pour la préparation d'une composition pharmaceutique destinée au traitement de la néoplasie, pour la cicatrisation, pour le traitement de la resténose, pour réguler l'hématopoïèse dans le développement de cellule endothéliale, pour stimuler une réponse immunitaire contre des infections parasitaires, bactériennes ou virales, ou pour le traitement et/ou la prévention de maladies autoimmunes.

17- Utilisation de l'antagoniste/inhibiteur selon la revendication 13 pour la préparation d'une composition pharmaceutique destinée au traitement de la cachexie, d'un accès pernicieux, de l'arthrite rhumatoïde, pour la prévention de rejet de greffe, pour inhiber la résorption osseuse, pour le traitement et/ou la prévention de l'ostéoporose, ou pour le traitement du choc endotoxique.

18- Procédé de diagnostic d'une maladie ou d'une susceptibilité à une maladie liée à une sous-expression du polypeptide selon la revendication 10 comprenant la détermination d'une mutation dans une séquence d'acide nucléique codant pour ledit polypeptide.

19- Procédé de diagnostic comprenant l'analyse de la présence du polypeptide selon la revendication 10 dans un échantillon dérivé d'un hôte.

20- Méthode d'identification de composés qui se lient et inhibent l'activation du polypeptide selon la revendication 10 comprenant :

- (a) mettre en contact une cellule exprimant à sa surface un récepteur au polypeptide, ledit récepteur étant associé à un second élément capable de produire un signal détectable en réponse à la liaison d'un composé audit récepteur, avec un polypeptide TNF delta analytiquement détectable et un composé dans des conditions permettant la liaison au récepteur ; et
- (b) déterminer si le composé se lie et inhibe le récepteur par la détection de l'absence d'un signal généré par l'interaction du TNF delta avec le récepteur.

EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

CLAIMS

Druckexemplar

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polypeptide encoded by a polynucleotide of any one of (a) to (d) wherein said fragment is capable of stimulating an immune response; ~~and~~ X
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence; X
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response; ~~and~~ X
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

VOSSIUS & PARTNER

EPO - Munich
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27. Juni 2003

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Partnerschaftsregister Amtsgericht München PR 89

EP 96 91 0483.5-2110
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Our Ref.: C 2276 EP

June 26, 2003
JAE/OOV

This is in reply to the Rule 51(4) Communication issued on February 17, 2003:

1. We request the correction of the following grammatical and typographical errors on page 1 of the claims set:

claim 1(f), last line: delete "and" after "...response;";

claim 1(g), last line: replace "." with ";"; and

claim 1(h), last line: insert "and" after "...response;".

We enclose correspondingly amended page 1 of the claims set.

2. Applicants declare their approval of the text as specified in the Rule 51(4) Communication provided that the amendments requested above will be accepted.
3. Furthermore, we herewith enclose the German and French translations of the claims as allowed.

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4. It is requested that 3 copies of the printed patent specification be supplied together with the Certificate for a European Patent.

5. Fees payable to the EPO:

- Fee for grant and printing (64 pages)	EUR 1.145,00
- Fee for 3 printed patent specifications	EUR 12,00
Total	<u>EUR 1.157,00</u>

The deduction of the total amount in EUR is to be made upon receipt of the enclosed debit order from our deposit account No. 2800.0321 at the EPO in Munich.

Zur Kasse



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosures:

Amended page 1 of the claims set
German claims, in dupl.
French claims, in dupl.
Order to debit account

EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

Druckexemplar

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polypeptide encoded by a polynucleotide of any one of (a) to (d) wherein said fragment is capable of stimulating an immune response; ~~and~~ X
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence; X
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response; ~~and~~ X
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON

This invention relates, in part, to newly identified polynucleotides and polypeptides; variants and derivatives of the polynucleotides and polypeptides; processes for making the polynucleotides and the polypeptides, and their variants and derivatives; agonists and antagonists of the polypeptides; and uses of the polynucleotides, polypeptides, variants, derivatives, agonists and antagonists. In particular, in these and in other regards, the invention relates to polynucleotides and polypeptides of human tumor necrosis factor delta and epsilon, sometimes hereinafter referred to as "TNF delta" and "TNF epsilon".

BACKGROUND OF THE INVENTION

Human tumor necrosis factors α (TNF- α) and β (TNF- β or lymphotoxin) are related members of a broad class of polypeptide mediators, which includes the interferons, interleukins and growth factors, collectively called cytokines (Beutler, B. and Cerami, A., *Annu. Rev. Immunol.*, 7:625-655, 1989).

Tumor necrosis factor (TNF- α and TNF- β) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine capable of numerous biological activities including apoptosis of some transformed cell lines, mediation of cell activation and proliferation and also as playing important roles in immune regulation and inflammation.

To date, there are nine known members of the TNF-ligand superfamily, TNF- α , TNF- β (lymphotoxin- α), LT- β , OX40L, FASL, CD30L, CD27L, CD40L and 4-1BBL. The ligands of the TNF ligand superfamily are acidic, TNF-like molecules with approximately 20% sequence homology in the extracellular domains (range, 12%-36%) and exist mainly as membrane-bound forms with the biologically active form being a trimeric/multimeric complex. Soluble forms of the TNF ligand superfamily have only been identified so far for TNF, LT α , and FASL (for a general review, see Gruss, H. and Dower, S.K., *Blood*, 85 (12):3378-3404 (1995)), which is hereby incorporated by reference in its entirety. *ESTs have been reported by Seed in EP-A1 330 191 (EST N 90606) and by Adams, Nature 355: 632-634 (EST M 78230). In addition, a genomic DNA fragment has been deposited under 260980.*

These proteins are involved in regulation of cell proliferation, activation, and differentiation, including control of cell survival or death by apoptosis or cytotoxicity (Armitage, R.J., *Curr. Opin. Immunol.*, 6:407 (1994) and Smith, C.A., *Cell*, 75:959 (1994).

TNF is produced by a number of cell types, including monocytes, fibroblasts, T cells, natural killer (NK) cells and predominately by activated macrophages. TNF- α has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, resistance to parasites, producing an anti-viral response, septic shock, growth regulation, vascular endothelium effects and metabolic effects. TNF- α also triggers endothelial cells to secrete various factors, including PAF-1, IL-1, GM-CSF and IL-6 to promote cell proliferation. In addition, TNF- α up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1.

The first step in the induction of the various cellular responses mediated by the members of the TNF ligand superfamily is their binding to specific cell surface receptors. The TNF receptor superfamily contains at present ten known membrane proteins and several viral open reading frames encoding TNFR-related molecules. The p75 low-affinity Nerve Growth Factor (NGF) receptor was the first cloned receptor of this family (Johnson, D. et al. *Cell*, 47:545 (1986)). Subsequently, cloning of two specific receptors for TNF show that they were related to the NGF receptor (Loetscher, H. et al., *Cell*, 61:351 (1990)). In recent years, a new type I-transmembrane TNF receptor superfamily has been established. This family includes the p75 nerve growth factor receptor, p60 TNFR-I, p80 TNFR-II, TNFR-RP/TNFR-III, CD27, CD30, CD40, 4-1BB, OX40 and FAS/APO-1. In addition, several viral open reading frames encoding soluble TNF receptors have been identified, such as SFV-T2 in Shope fibroma virus (Smith, C.A. et al., *Biochem. Biophys. Res. Commun.*, 176:335, 1991) and Va53 or SaIF19R in vaccinia virus (Howard, S.T., *Virology*, 180:633, 1991). These receptors are characterized by multiple cysteine-rich domains in the extracellular (amino-terminal) domain, which have been shown to be involved in ligand binding. The average homology in the cysteine-rich extracellular region between the human family members are in the range of 25 to 30%.

Clearly, there is a need for factors that regulate activation, and differentiation of normal and abnormal cells. There is a need, therefore, for identification and characterization of such factors that modulate activation and differentiation of cells, both normally and in disease states. In particular, there is a need to isolate and characterize additional TNF ligands akin to members of the TNF ligand super-family that control apoptosis of transformed cell lines, mediate cell activation and proliferation and are functionally linked as primary mediators of immune regulation and inflammatory response, and, among other things, can play a role in preventing, ameliorating or correcting dysfunctions or diseases.

SUMMARY OF THE INVENTION

Toward these ends, and others, it is an object of the present invention to provide novel polypeptides, referred to as novel TNF delta and TNF epsilon which have been putatively identified as being tumor necrosis factor ligands by homology between the amino acid sequence set out in Figures 1 and 2 and known amino acid sequences of other proteins in the tumor necrosis factor family such as human TNF α and TNF β .

The polypeptides of the present invention have been identified as a novel members of the TNF ligand super-family based on structural and biological similarities.

It is a further object of the invention, moreover, to provide polynucleotides that encode TNF delta and TNF epsilon, particularly polynucleotides that encode the polypeptide herein designated TNF delta and TNF epsilon.

In a particularly preferred embodiment of this aspect of the invention the polynucleotides comprise the region encoding human TNF delta and TNF epsilon in the sequences set out in Figures 1 and 2.

In accordance with this aspect of the invention there are provided isolated nucleic acid molecules encoding human TNF delta, including mRNAs, cDNAs, genomic DNAs and, in further embodiments of this aspect of the invention, biologically, diagnostically, clinically or therapeutically useful variants, analogs or derivatives thereof, or fragments thereof, including fragments of the variants, analogs and derivatives.

Among the particularly preferred embodiments of this aspect of the invention are naturally occurring allelic variants of human TNF delta and TNF epsilon.

In accordance with this aspect of the present invention there are provided isolated nucleic acid molecules encoding a mature human TNF delta polypeptide expressed by

the human cDNA contained in ATCC Deposit No. 97377 deposited on December 8, 1995 and a mature human TNF epsilon polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97457 deposited on March 1, 1996.

It also is an object of the invention to provide TNF delta polypeptides, particularly human TNF delta and TNF epsilon polypeptides, that destroy some transformed cell lines, mediate cell activation and proliferation and are functionally linked as primary mediators of immune regulation and inflammatory response.

In accordance with this aspect of the invention there are provided novel polypeptides of human origin referred to herein as TNF delta and TNF epsilon as well as biologically, diagnostically or therapeutically useful fragments, variants and derivatives thereof, variants and derivatives of the fragments, and analogs of the foregoing.

Among the particularly preferred embodiments of this aspect of the invention are variants of human TNF delta and TNF epsilon encoded by naturally occurring alleles of the human TNF delta and TNF epsilon gene.

It is another object of the invention to provide a process for producing the aforementioned polypeptides, polypeptide fragments, variants and derivatives, fragments of the variants and derivatives, and analogs of the foregoing. In a preferred embodiment of this aspect of the invention there are provided methods for producing the aforementioned TNF delta and TNF epsilon polypeptides comprising culturing host cells having expressibly incorporated therein an exogenously-derived human TNF delta-encoding polynucleotide and TNF epsilon-encoding polynucleotide under conditions for expression of human TNF delta and TNF epsilon in the host and then recovering the expressed polypeptide.

In accordance with another object the invention there are provided products, compositions, processes and methods that utilize the aforementioned polypeptides and polynucleotides for research, biological, clinical and therapeutic purposes, *inter alia*.

In accordance with certain preferred embodiments of this aspect of the invention, there are provided products, compositions and methods, *inter alia*, for, among other things: assessing TNF delta and TNF epsilon expression in cells by determining TNF delta and TNF epsilon polypeptides or TNF delta-encoding mRNA or TNF epsilon-encoding mRNA polypeptides; assaying genetic variation and aberrations, such as defects, in TNF delta and TNF epsilon genes; and administering a TNF delta or TNF epsilon polypeptide or polynucleotide to an organism to augment TNF delta or TNF epsilon function or remediate TNF delta or TNF epsilon dysfunction.

In accordance with certain preferred embodiments of this and other aspects of the invention there are provided polynucleotides and in particular probes that hybridize to human TNF delta or TNF epsilon sequences.

In certain additional preferred embodiments of this aspect of the invention there are provided antibodies against TNF delta or TNF epsilon polypeptides. In certain particularly preferred embodiments in this regard, the antibodies are highly selective for human TNF delta or TNF epsilon.

In accordance with another aspect of the present invention, there are provided TNF delta or TNF epsilon agonists. Among preferred agonists are molecules that mimic TNF delta or TNF epsilon, that bind to TNF delta-binding molecules or receptor molecules or to TNF epsilon-binding molecules or receptor molecules, and that elicit or augment TNF delta-induced or TNF epsilon-induced responses. Also among preferred agonists are molecules that interact with TNF delta and TNF epsilon or TNF delta and TNF epsilon polypeptides, or with other modulators of TNF delta activities,

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and thereby potentiate or augment an effect of TNF delta and TNF epsilon or more than one effect of TNF delta and TNF epsilon.

In accordance with yet another aspect of the present invention, there are provided TNF delta and TNF epsilon antagonists. Among preferred antagonists are those which mimic TNF delta and TNF epsilon so as to bind to TNF delta and TNF epsilon receptors or binding molecules but not elicit a TNF delta- and TNF epsilon-induced response or more than one TNF delta- and TNF epsilon-induced response. Also among preferred antagonists are molecules that bind to or interact with TNF delta and TNF epsilon so as to inhibit an effect of TNF delta and TNF epsilon or more than one effect of TNF delta and TNF epsilon or which prevent expression of TNF delta and TNF epsilon.

The agonists and antagonists may be used to mimic, augment or inhibit the action of TNF delta and TNF epsilon polypeptides. They may be used, for instance, to prevent septic shock, inflammation, cerebral malaria, activation of the HIV virus, graft-host rejection, bone resorption, rheumatoid arthritis and cachexia.

In a further aspect of the invention there are provided compositions comprising a TNF delta and TNF epsilon polynucleotide or a TNF delta and TNF epsilon polypeptide for administration to cells in vitro, to cells ex vivo and to cells in vivo, or to a multicellular organism. In certain particularly preferred embodiments of this aspect of the invention, the compositions comprise a TNF delta and TNF epsilon polynucleotide for expression of a TNF delta and TNF epsilon polypeptide in a host organism for treatment of disease. Particularly preferred in this regard is expression in a human patient for treatment of a dysfunction associated with aberrant endogenous activity of TNF delta and TNF epsilon.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill from the following description. It should be

understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. ~~Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.~~

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings depict certain embodiments of the invention. They are illustrative only and do not limit the invention otherwise disclosed herein.

Figure 1 shows the nucleotide and deduced amino acid sequence of human TNF delta.

Figure 2 shows the nucleotide and deduced amino acid sequence of human TNF epsilon.

Figure 3 shows the regions of similarity (alignment report) between amino acid sequences of TNF α , TNF β , TNF δ and TNF ϵ polypeptides.

Figure 4 shows structural and functional features of TNF delta deduced by the indicated techniques, as a function of amino acid sequence.

Figure 5 shows structural and functional features of TNF epsilon deduced by the indicated techniques, as a function of amino acid sequence.

The following illustrative explanations are provided to facilitate understanding of certain terms used frequently herein, particularly in the examples. The explanations are provided as a convenience and are not limitative of the invention.

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The term "digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes referred to herein are commercially available and their reaction conditions, cofactors and other requirements for use are known and routine to the skilled artisan.

For analytical purposes, typically, 1 μ g of plasmid or DNA fragment is digested with about 2 units of enzyme in about 20 μ l of reaction buffer. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μ g of DNA are digested with 20 to 250 units of enzyme in proportionately larger volumes.

Appropriate buffers and substrate amounts for particular restriction enzymes are described in standard laboratory manuals, such as those referenced below, and they are specified by commercial suppliers.

Incubation times of about 1 hour at 37°C are ordinarily used, but conditions may vary in accordance with standard procedures, the supplier's instructions and the particulars of the reaction. After digestion, reactions may be analyzed, and fragments may be purified by electrophoresis through an agarose or polyacrylamide gel, using well known methods that are routine for those skilled in the art.

The term "genetic element" generally means a polynucleotide comprising a region that encodes a polypeptide or a region that regulates transcription or translation or other processes important to expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression.

Genetic elements may be comprised within a vector that replicates as an episomal element; that is, as a molecule physically independent of the host cell genome. They may be comprised within mini-chromosomes, such as those that arise during

amplification of transfected DNA by methotrexate selection in eukaryotic cells. Genetic elements also may be comprised within a host cell genome; not in their natural state but, rather, following manipulation such as isolation, cloning and introduction into a host cell in the form of purified DNA or in a vector, among others.

The term "isolated" means altered "by the hand of man" from its natural state; i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. For example, with respect to polynucleotides, the term isolated means that it is separated from the chromosome and cell in which it naturally occurs.

As part of or following isolation, such polynucleotides can be joined to other polynucleotides, for mutagenesis, to form fusion proteins, and for propagation or expression in a host, for instance. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells, in culture or in whole organisms, after which such DNAs still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment.

Similarly, the polynucleotides and polypeptides may occur in a composition, such as a media formulations, solutions for introduction of polynucleotides or polypeptides, for example, into cells, compositions or solutions for chemical or enzymatic reactions, for instance, which are not naturally occurring compositions, and, therein remain isolated polynucleotides or polypeptides within the meaning of that term as it is employed herein.

The term "ligation" refers to the process of forming phosphodiester bonds between two or more polynucleotides, which most often are double stranded DNAs.

Techniques for ligation are well known to the art and protocols for ligation are described in standard laboratory manuals and references, such as, for instance, Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) and Maniatis et al., pg. 146, as cited below.

The term "oligonucleotide(s)" refers to relatively short polynucleotides. Often the term refers to single-stranded deoxyribonucleotides, but it can refer as well to single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs, among others.

Oligonucleotides, such as single-stranded DNA probe oligonucleotides, often are synthesized by chemical methods, such as those implemented on automated oligonucleotide synthesizers. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

Initially, chemically synthesized DNAs typically are obtained without a 5' phosphate. The 5' ends of such oligonucleotides are not substrates for phosphodiester bond formation by ligation reactions that employ DNA ligases typically used to form recombinant DNA molecules. Where ligation of such oligonucleotides is desired, a phosphate can be added by standard techniques, such as those that employ a kinase and ATP.

The 3' end of a chemically synthesized oligonucleotide generally has a free hydroxyl group and, in the presence of a ligase, such as T4 DNA ligase, readily will form a phosphodiester bond with a 5' phosphate of another polynucleotide, such as another oligonucleotide. As is well known, this reaction can be prevented selectively, where desired, by removing the 5' phosphates of the other polynucleotide(s) prior to ligation.

Plasmids generally are designated herein by a lower case p preceded and/or followed by capital letters and/or numbers, in accordance with standard naming conventions that are familiar to those of skill in the art. Starting plasmids disclosed herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids by routine application of well known, published procedures. Many plasmids and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art. Moreover, those of skill readily may construct any number of other plasmids suitable for use in the invention. The properties, construction and use of such plasmids, as well as other vectors, in the present invention will be readily apparent to those of skill from the present disclosure.

The term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or

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modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, *inter alia*.

The term "polypeptides," as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types.

It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present invention are, to name an illustrative few, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin,

covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, *Analysis for protein modifications and nonprotein cofactors. Meth. Enzymol.*, 182: 626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci.*, 663: 48-62 (1992).

It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslation events, including natural processing event, and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural process and by entirely synthetic methods, as well.

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Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli*, prior to proteolytic processing, almost invariably will be N-formylmethionine.

The modifications that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications in large part will be determined by the host cell posttranslational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out the same posttranslational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to express efficiently mammalian proteins having native patterns of glycosylation, *inter alia*. Similar considerations apply to other modifications. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. In general, as used herein, the term polypeptide encompasses all such modifications, particularly those that are present in polypeptides synthesized by expressing a polynucleotide in a host cell.

The term "variant(s)" of polynucleotides or polypeptides, as the term is used herein, are polynucleotides or polypeptides that differ from a reference polynucleotide or polypeptide, respectively. Variants in this sense are described below and elsewhere in the present disclosure in greater detail.

A polynucleotide variant is a polynucleotide that differs in nucleotide sequence from another, reference polynucleotide. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. As noted below, changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Also as noted below, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below.

A polypeptide variant is a polypeptide that differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference and the variant are closely similar overall and, in many region, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

The term "receptor molecule," as used herein, refers to molecules which bind or interact specifically with TNF delta or TNF epsilon polypeptides of the present invention, including not only classic receptors, which are preferred, but also other molecules that specifically bind to or interact with polypeptides of the invention (which also may be referred to as "binding molecules" and "interaction molecules," respectively and as "TNF delta binding molecules" and "TNF delta interaction molecules" or "TNF epsilon binding molecules" and "TNF epsilon interaction molecules." Binding between polypeptides of the invention and such molecules, including receptor or binding or interaction molecules may be exclusive to polypeptides of the invention, which is very highly preferred, or it may be highly specific for polypeptides of the invention, which is highly preferred, or it may be highly specific to a group of proteins that includes

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polypeptides of the invention, which is preferred, or it may be specific to several groups of proteins at least one of which includes polypeptides of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel TNF delta and TNF epsilon polypeptides and polynucleotides, among other things, as described in greater detail below. In particular, the invention relates to polypeptides and polynucleotides which are related by amino acid sequence homology to the TNF ligand superfamily. The invention relates especially to TNF delta having the nucleotide and amino acid sequences set out in Figure 1, and to the TNF nucleotide and amino acid sequences of the human cDNA in ATCC Deposit No. 97377. The invention also relates especially to TNF epsilon having the nucleotide and amino acid sequences set out in Figure 2, and to the TNF epsilon nucleotide and amino acid sequences of the human cDNA in ATCC Deposit No. 97457. The deposits are hereinafter referred to as the deposited clones or as "the cDNA of the deposited clones." It will be appreciated that the nucleotide and amino acid sequences set out in Figures 1 and 2 were obtained by sequencing the human cDNA of the deposited clones. Hence, the sequence of the deposited clone is controlling as to any discrepancies between the two and any reference to the sequences of Figures 1 and 2 include reference to the sequences of the human cDNA's of the deposited clones.

In accordance with one aspect of the present invention, there are provided isolated polynucleotides which encode the TNF delta and TNF epsilon polypeptides having the deduced amino acid sequences of Figures 1 and 2.

Using the information provided herein, such as the polynucleotide sequence set out in Figure 1, a polynucleotide of the present invention encoding human TNF delta polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA from cells of human tissue as starting material. Illustrative of the invention, the polynucleotide set out in Figure 1 was discovered in a cDNA library derived from cells of human heart tissue.

Human TNF delta of the invention is structurally related to other proteins of the TNF ligand superfamily, as shown by the results of sequencing the cDNA encoding human TNF delta in the deposited clone. The cDNA sequence thus obtained is set out in Figure 1. It contains an open reading frame encoding a protein of about 233 amino acid residues with a deduced molecular weight of about 25.871 kDa. The protein exhibits greatest homology to TNF α , among known proteins. The entire amino acid sequence of TNF delta of Figure 1 has about 38% identity to the amino acid sequence of TNF α .

A polynucleotide of the present invention encoding human TNF epsilon polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA from cells of human tissue as starting material. Illustrative of the invention, the polynucleotide set out in Figure 2 was discovered in a cDNA library derived from cells of human heart tissue.

Human TNF epsilon of the invention is structurally related to other proteins of the TNF ligand superfamily, as shown by the results of sequencing the cDNA encoding human TNF epsilon in the deposited clone. The cDNA sequence thus obtained is set out in Figure 2. The TNF epsilon sequence is nearly identical to the sequence of TNF delta as set out in Figure 1 minus the initial 50 amino acids and a region of TNF delta comprising amino acid 86 to amino acid 92. Accordingly, TNF epsilon is a splicing variant of TNF delta. TNF epsilon comprises 168 amino acid residues and the sequence of Figure 2 shows the mature protein of TNF epsilon without any N-terminal hydrophobic region. The protein exhibits greatest homology to TNF α . TNF epsilon of Figure 2 has about 20% identity to the amino acid sequence of TNF α .

Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded DNA

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may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

The coding sequence which encodes the polypeptide may be identical to the coding sequence of the polynucleotide shown in Figures 1 and 2. It also may be a polynucleotide with a different sequence, which, as a result of the redundancy (degeneracy) of the genetic code, encodes the polypeptide of the DNA of Figures 1 and 2.

Polynucleotides of the present invention which encode the polypeptide of Figures 1 and 2 may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences; such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities.

Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in the pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA*, 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of

influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell*, 37:767 (1984), for instance.

In accordance with the foregoing, the term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides which include a sequence encoding a polypeptide of the present invention, particularly the human TNF delta and TNF epsilon having the amino acid sequences set out in Figures 1 and 2. The term encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by introns) together with additional regions, that also may contain coding and/or non-coding sequences.

The present invention further relates to variants of the herein above described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figures 1 and 2. A variant of the polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms.

Among variants in this regard are variants that differ from the aforementioned polynucleotides by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Among the particularly preferred embodiments of the invention in this regard are polynucleotides encoding polypeptides having the amino acid sequence of TNF delta and TNF epsilon set out in Figures 1 and 2; variants, analogs, derivatives and fragments thereof, and fragments of the variants, analogs and derivatives.

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Further particularly preferred in this regard are polynucleotides encoding TNF delta and TNF epsilon which have the amino acid sequence of the TNF delta and TNF epsilon polypeptide of Figures 1 and 2 in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNF delta and TNF epsilon. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polynucleotide encoding polypeptides having the amino acid sequence of Figures 1 and 2, without substitutions. Further preferred embodiments of the invention are polynucleotides that are at least 70% identical to a polynucleotide encoding the TNF delta and TNF epsilon polypeptide having the amino acid sequence set out in Figures 1 and 2, and polynucleotides which are complementary to such polynucleotides. Alternatively, most highly preferred are polynucleotides that comprise a region that is at least 80% identical to a polynucleotide encoding the TNF delta and TNF epsilon polypeptide and polynucleotides complementary thereto. In this regard, polynucleotides at least 90% identical to the same are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

Particularly preferred embodiments in this respect, moreover, are polynucleotides which encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figures 1 and 2.

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means

hybridization will occur when at least 95% and preferably at least 97% of the bases between sequences are complementary (e.g., G:C; A:T).

As discussed additionally herein regarding polynucleotide assays of the invention, for instance, polynucleotides of the invention as discussed above, may be used as a hybridization probe for cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding TNF delta and TNF epsilon and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the human TNF delta and TNF epsilon gene. Such probes generally will comprise at least 15 bases. Preferably, such probes will have at least 30 bases and may have at least 50 bases.

For example, the coding region of the TNF delta and TNF epsilon gene may be isolated by screening using the known DNA sequence to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the present invention is then used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to human disease, as further discussed herein relating to polynucleotide assays, *inter alia*.

The polynucleotides may encode a polypeptide which is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, may facilitate protein trafficking, may prolong or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in situ*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

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A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. When prosequences are removed such inactive precursors generally are activated. Some or all of the prosequences may be removed before activation. Generally, such precursors are called proproteins.

In sum, a polynucleotide of the present invention may encode a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences which are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

Deposits containing human TNF delta and human TNF epsilon cDNA have been deposited with the American Type Culture Collection, as noted above. Also as noted above, the cDNA deposit is referred to herein as "the deposited clone" or as "the cDNA of the deposited clone." The clones were deposited with the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, USA, on December 8, 1995 and March 1, 1996, and assigned ATCC Deposit No. 97377 and 97457, respectively. The deposited materials are pBluescript SK (-) plasmids (Stratagene, La Jolla, CA) that contains the full length TNF delta and TNF epsilon human cDNA.

The deposits have been made under the terms of the Budapest Treaty on the international recognition of the deposit of micro-organisms for purposes of patent procedure. The strains will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited material, as well as the amino acid

sequence of the polypeptide encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to human TNF delta and TNF epsilon polypeptides having the deduced amino acid sequences of Figures 1 and 2. The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide. In certain preferred embodiments it is a recombinant polypeptide.

The invention also relates to fragments, analogs and derivatives of these polypeptides. The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figures 1 and 2 means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analog of the polypeptide of Figures 1 and 2 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

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Among the particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of TNF delta and TNF epsilon set out in Figures 1 and 2, variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments. Alternatively, particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of the TNF delta and TNF epsilon of the human cDNA in the deposited clone, variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments.

Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Further particularly preferred in this regard are variants, analogs, derivatives and fragments, and variants, analogs and derivatives of the fragments, having the amino acid sequence of the TNF delta and TNF epsilon polypeptide of Figures 1 and 2 or of the cDNA in the deposited clone, in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNF delta and TNF epsilon. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polypeptides having the amino acid sequence of Figures 1 and 2 without substitutions.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The TGF delta polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

The TGF epsilon polypeptides of the present invention include the polypeptide of SEQ ID NO:4 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:4 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:4 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:4 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length

polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

A fragment is a polypeptide having an amino acid sequence that entirely is the same as part but not all of the amino acid sequence of the aforementioned TNF delta and TNF epsilon polypeptides and variants or derivatives thereof. Such fragments may be "free-standing," i.e., not part of or fused to other amino acids or polypeptides, or they may be comprised within a larger polypeptide of which they form a part or region. When comprised within a larger polypeptide, the presently discussed fragments most preferably form a single continuous region. However, several fragments may be comprised within a single larger polypeptide. For instance, certain preferred embodiments relate to a fragment of a TNF delta and TNF epsilon polypeptide of the present comprised within a precursor polypeptide designed for expression in a host and having heterologous pre and pro-polypeptide regions fused to the amino terminus of the TNF delta and TNF epsilon fragment and an additional region fused to the carboxyl terminus of the fragment. Therefore, fragments in one aspect of the meaning intended herein, refers to the portion or portions of a fusion polypeptide or fusion protein derived from TNF delta and TNF epsilon.

As representative examples of polypeptide fragments of the invention, there may be mentioned those which have from about 30 to about 233 amino acids. In this context, "about" includes the particularly recited range and ranges larger or smaller by several, a few, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes. For instance, about 100 to 233 amino acids in this context means a polypeptide fragment of 100 plus or minus several, a few, 5, 4, 3, 2 or 1 amino acids to 233 plus or minus several a few, 5, 4, 3, 2 or 1 amino acid residues, i.e., ranges as broad as 100 minus several amino acids to 233 plus several amino acids to as narrow as 100 plus several amino acids to 233 minus several amino acids.

Highly preferred in this regard are the recited ranges plus or minus as many as 5 amino acids at either or at both extremes. Particularly highly preferred are the recited ranges plus or minus as many as 3 amino acids at either or at both the recited extremes. Especially particularly highly preferred are ranges plus or minus 1 amino acid at either or at both extremes or the recited ranges with no additions or deletions. Most highly preferred of all in this regard are fragments from about 15 to about 233 amino acids.

Among especially preferred fragments of the invention are truncation mutants of TNF delta and TNF epsilon. Truncation mutants include TNF delta and TNF epsilon polypeptides having the amino acid sequence of Figures 1 and 2, or of variants or derivatives thereof, except for deletion of a continuous series of residues (that is, a continuous region, part or portion) that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or, as in double truncation mutants, deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Fragments having the size ranges set out about also are preferred embodiments of truncation fragments, which are especially preferred among fragments generally.

Also preferred in this aspect of the invention are fragments characterized by structural or functional attributes of TNF delta and TNF epsilon. Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet-forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions and high antigenic index regions of TNF delta and TNF epsilon.

Certain preferred regions in these regards are set out in Figure 4 for TNF delta and Figure 5 for TNF epsilon, and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in Figures

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1 and 2. As set out in Figures 4 and 5, such preferred regions include Garnier-Robson alpha-regions, beta-regions, turn-regions and coil-regions, Chou-Fasman alpha-regions, beta-regions and turn-regions, Kyte-Doolittle hydrophilic regions and hydrophilic regions, Eisenberg alpha and beta amphipathic regions, Karplus-Schulz flexible regions, Emmini surface-forming regions and Jameson-Wolf high antigenic index regions.

Among highly preferred fragments in this regard are those that comprise regions of TNF delta and TNF epsilon that combine several structural features, such as several of the features set out above. In this regard, the regions defined by the residues following the signal peptide region of Figures 1, 2, 4 and 5, which all are characterized by amino acid compositions highly characteristic of turn-regions, hydrophilic regions, flexible-regions, surface-forming regions, and high antigenic index-regions, are especially highly preferred regions. Such regions may be comprised within a larger polypeptide or may be by themselves a preferred fragment of the present invention, as discussed above. It will be appreciated that the term "about" as used in this paragraph has the meaning set out above regarding fragments in general.

Further preferred regions are those that mediate activities of TNF delta and TNF epsilon. Most highly preferred in this regard are fragments that have a chemical, biological or other activity of TNF delta and TNF epsilon, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Highly preferred in this regard are fragments that contain regions that are homologs in sequence, or in position, or in both sequence and to active regions of related polypeptides, such as the related polypeptides set out in Figure 3, including human TNF α and β . Among particularly preferred fragments in these regards are truncation mutants, as discussed above.

It will be appreciated that the invention also relates to, among others, polynucleotides encoding the aforementioned fragments, polynucleotides that hybridize to polynucleotides encoding the fragments, particularly those that hybridize under

stringent conditions, and polynucleotides, such as PCR primers, for amplifying polynucleotides that encode the fragments. In these regards, preferred polynucleotides are those that correspondent to the preferred fragments, as discussed above.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate polynucleotides and express polypeptides of the present invention. For instance, polynucleotides may be introduced into host cells using well known techniques of infection, transduction, transfection, transvection and transformation. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention. Thus, for instance, polynucleotides of the invention may be transfected into host cells with another, separate, polynucleotide encoding a selectable marker, using standard techniques for co-transfection and selection in, for instance, mammalian cells. In this case the polynucleotides generally will be stably incorporated into the host cell genome.

Alternatively, the polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. The vector construct may be introduced into host cells by the aforementioned techniques. Generally, a plasmid vector is introduced as DNA in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. Electroporation also may be used to introduce polynucleotides into a host. If the vector is a virus, it may be packaged in vitro or introduced into a packaging cell and the packaged virus may be transduced into cells. A wide variety of techniques suitable for making polynucleotides and for introducing polynucleotides into cells in accordance with this aspect of the invention are well known and routine to those of skill in the art. Such techniques are reviewed at length in

Sambrook *et al.* cited above, which is illustrative of the many laboratory manuals that detail these techniques. In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsidated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.

The engineered host cells can be cultured in conventional nutrient media, which may be modified as appropriate for, *inter alia*, activating promoters, selecting transformants or amplifying genes. Culture conditions, such as temperature, pH and the

like, previously used with the host cell selected for expression generally will be suitable for expression of polypeptides of the present invention as will be apparent to those of skill in the art.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques. In general, a DNA sequence for expression is joined to an expression vector by cleaving the DNA sequence and the expression vector with one or more restriction endonucleases and then joining the restriction fragments together using T4 DNA ligase. Procedures for restriction and ligation that can be used to this end are well known and routine to those of skill. Suitable procedures in this regard, and for constructing expression vectors using alternative techniques, which also are well known and routine to those skill, are set forth in great detail in Sambrook et al. cited elsewhere herein.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. It will

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be understood that numerous promoters not mentioned are suitable for use in this aspect of the invention are well known and readily may be employed by those of skill in the manner illustrated by the discussion and the examples herein.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera Sf9* cells; animal cells such as CHO, COS and Bowes melanoma cells;

and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

More particularly, the present invention also includes recombinant constructs, such as expression constructs, comprising one or more of the sequences described above. The constructs comprise a vector, such as a plasmid or viral vector, into which such a sequence of the invention has been inserted. The sequence may be inserted in a forward or reverse orientation. In certain preferred embodiments in this regard, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and there are many commercially available vectors suitable for use in the present invention.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide of the invention in a host may be used in this aspect of the invention.

Promoter regions can be selected from any desired gene using vectors that contain a reporter transcription unit lacking a promoter region, such as a

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chloramphenicol acetyl transferase ("cat") transcription unit, downstream of restriction site or sites for introducing a candidate promoter fragment; i.e., a fragment that may contain a promoter. As is well known, introduction into the vector of a promoter-containing fragment at the restriction site upstream of the cat gene engenders production of CAT activity, which can be detected by standard CAT assays. Vectors suitable to this end are well known and readily available. Two such vectors are pKK232-8 and pCM7. Thus, promoters for expression of polynucleotides of the present invention include not only well known and readily available promoters, but also promoters that readily may be obtained by the foregoing technique, using a reporter gene.

Among known bacterial promoters suitable for expression of polynucleotides and polypeptides in accordance with the present invention are the *E. coli* lacI and lacZ and promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR, PL promoters and the trp promoter. Among known eukaryotic promoters suitable in this regard are the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter. Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods

are described in many standard laboratory manuals, such as Davis *et al.* Basic Methods in Molecular Biology, (1986). Constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Generally, recombinant expression vectors will include origins of replication, a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector. Among suitable promoters are those derived from the genes that encode glycolytic enzymes such as 3-phosphoglycerate kinase ("PGK"), a-factor, acid phosphatase, and heat shock proteins, among others. Selectable markers include the ampicillin resistance gene of *E. coli* and the *trp1* gene of *S. cerevisiae*.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

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Polynucleotides of the invention, encoding the heterologous structural sequence of a polypeptide of the invention generally will be inserted into the vector using standard techniques so that it is operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed. Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiating AUG. Also, generally, there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal and a transcription termination signal appropriately disposed at the 3' end of the transcribed region.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

Suitable prokaryotic hosts for propagation, maintenance or expression of polynucleotides and polypeptides in accordance with the invention include *Escherichia*

coli, *Bacillus subtilis* and *Salmonella typhimurium*. Various species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus* are suitable hosts in this regard. Moreover, many other hosts also known to those of skill may be employed in this regard.

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, where the selected promoter is inducible it is induced by appropriate means (*e.g.*, temperature shift or exposure to chemical inducer) and cells are cultured for an additional period. Cells typically then are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can be employed for expression, as well. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblast, described in Gluzman *et al.*, *Cell*, 23:175 (1981). Other cell lines capable of expressing a compatible vector include for example, the C127, 3T3, CHO, HeLa, human kidney 293 and BHK cell lines.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation

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sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression. In certain preferred embodiments in this regard DNA sequences derived from the SV40 splice sites, and the SV40 polyadenylation sites are used for required non-transcribed genetic elements of these types.

The polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

The polynucleotides and polypeptides of the present invention may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties TNF delta and TNF epsilon. Among these are applications in apoptosis of transformed cell lines, mediation of cell activation and proliferation and primary mediators of immune regulation antimicrobial,

antiviral and inflammatory response susceptibility to pathogens. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are illustrated further by the following discussion.

This invention is also related to the use of the polynucleotides of the present invention to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of a polypeptide of the present invention associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of polypeptide of the present invention, such as, for example, neoplasia such as tumors.

Individuals carrying mutations in a gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. PCR (Saiki *et al.*, *Nature*, 324: 163-166 1986). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding TNF delta or TNF epsilon can be used to identify and analyze TNF delta or TNF epsilon expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled TNF delta or TNF epsilon RNA or alternatively, radiolabeled TNF delta or TNF epsilon antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such

methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science*, 230:1242 1985).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci., USA*, 85:4397-4401, 1985). Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA. In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according

to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a gene of the present invention. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA the is used for *in situ* chromosome mapping using well known techniques for this purpose. Typically, in accordance with routine procedures for chromosome mapping, some trial and error may be necessary to identify a genomic probe that gives a good *in situ* hybridization signal.

In some cases, in addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this

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technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The present invention also relates to a diagnostic assays such as quantitative and diagnostic assays for detecting levels of a protein in the present invention in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of TNF protein of the present invention compared to normal control tissue samples may be used to detect the presence of neoplasia, for example. Assay techniques that can be used to determine levels of a protein, such as a protein of the present invention, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and

ELISA assays. Among these ELISAs frequently are preferred. An ELISA assay initially comprises preparing an antibody specific to a protein of the present invention, preferably a monoclonal antibody. In addition a reporter antibody generally is prepared which binds to the monoclonal antibody. The reporter antibody is attached to a detectable reagent such as radioactive, fluorescent or enzymatic, which in this example is horseradish peroxidase enzyme.

To carry out an ELISA assay a sample is removed from a host and incubated on a solid support, *e.g.* a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any protein of the present invention attached to the polystyrene dish. Unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to a protein of the present invention. Unattached reporter antibody is then washed out. Reagents for peroxidase activity, including a colorimetric substrate are then added to the dish. Immobilized peroxidase, linked to protein of the present invention through the primary and secondary antibodies, produces a colored reaction product. The amount of color developed in a given time period indicates the amount of protein of the present invention present in the sample. Quantitative results typically are obtained by reference to a standard curve.

A competition assay may be employed wherein antibodies specific to protein of the present invention attached to a solid support and labeled protein of the present invention and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of protein of the present invention in the sample.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., *Nature*, 256:495-497 (1975), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today*, 4:72 (1983) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, pg. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985).

Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or purify the polypeptide of the present invention by attachment of the antibody to a solid support for isolation and/or purification by affinity chromatography.

Thus, the polypeptides of the present invention of the present invention may be employed to inhibit neoplasia, such as tumor cell growth. The polypeptides of the present invention may be responsible for tumor destruction through apoptosis and cytotoxicity to certain cells. The polypeptides of the present invention also induce up-regulation of adhesion cells, for example, LFA-1, therefore, may be employed for wound-healing. The polypeptides of the present invention may also be employed to treat diseases which require growth promotion activity, for example, restenosis, since the polypeptides of the present invention have proliferation effects on cells of endothelial origin. The polypeptides of the present invention may, therefore, also be employed to regulate hematopoiesis in endothelial cell development.

The polypeptides of the present invention also stimulate the activation of T-cells, and may, therefore, be employed to stimulate an immune response against a variety of parasitic, bacterial and viral infections. The polypeptides of the present invention may also be employed in this respect to eliminate autoreactive T-cells to treat and/or prevent autoimmune diseases. An example of an autoimmune disease is Type I diabetes.

This invention also provides a method for identification of molecules, such as receptor molecules, that bind the proteins of the present invention. Genes encoding proteins that bind the proteins of the present invention, such as receptor proteins, can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. Such methods are described in many laboratory manuals such as, for instance, Coligan *et al.*, Current Protocols in Immunology 1(2): Chapter 5 (1991).

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For instance, expression cloning may be employed for this purpose. To this end polyadenylated RNA is prepared from a cell responsive to the proteins of the present invention, a cDNA library is created from this RNA, the library is divided into pools and the pools are transfected individually into cells that are not responsive to the proteins of the present invention. The transfected cells then are exposed to labeled the proteins of the present invention. The proteins of the present invention can be labeled by a variety of well-known techniques including standard methods of radio-iodination or inclusion of a recognition site for a site-specific protein kinase. Following exposure, the cells are fixed and binding of cytostatin is determined. These procedures conveniently are carried out on glass slides.

Pools are identified of cDNA that produced TNF delta or TNF epsilon binding cells. Sub-pools are prepared from these positives, transfected into host cells and screened as described above. Using an iterative sub-pooling and re-screening process, one or more single clones that encode the putative binding molecule, such as a receptor molecule, can be isolated.

Alternatively a labeled ligand can be photoaffinity linked to a cell extract, such as a membrane or a membrane extract, prepared from cells that express a molecule that it binds, such as a receptor molecule. Cross-linked material is resolved by polyacrylamide gel electrophoresis ("PAGE") and exposed to X-ray film. The labeled complex containing the ligand-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing can be used to design unique or degenerate oligonucleotide probes to screen cDNA libraries to identify genes encoding the putative receptor molecule.

Polypeptides of the invention also can be used to assess TNF delta or TNF epsilon binding capacity of TNF delta or TNF epsilon binding molecules, such as receptor molecules, in cells or in cell-free preparations.

The invention also provides a method of screening compounds to identify those which enhance or block the action of TNF delta or TNF epsilon on cells, such as its interaction with TNF delta or TNF epsilon binding molecules such as receptor molecules. An agonist is a compound which increases the natural biological functions of polypeptides of the present invention or which functions in a manner similar to polypeptides of the present invention, while antagonists decrease or eliminate such functions.

For example, a cellular compartment, such as a membrane or a preparation thereof, such as a membrane-preparation, may be prepared from a cell that expresses a molecule that binds TNF delta or TNF epsilon, such as a molecule of a signaling or regulatory pathway modulated by TNF delta or TNF epsilon. The preparation is incubated with labeled TNF delta or TNF epsilon in the absence or the presence of a candidate molecule which may be a TNF delta or TNF epsilon agonist or antagonist. The ability of the candidate molecule to bind the binding molecule is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, i.e., without inducing the effects of TNF delta or TNF epsilon on binding the TNF delta or TNF epsilon binding molecule, are most likely to be good antagonists. Molecules that bind well and elicit effects that are the same as or closely related to TNF delta or TNF epsilon are agonists.

TNF delta or TNF epsilon-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a second messenger system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of TNF delta or TNF epsilon or molecules that elicit the same effects as TNF delta or TNF epsilon. Second messenger systems that may be useful in this regard include but are not limited to AMP guanylate cyclase, ion channel or phosphoinositide hydrolysis second messenger systems.

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Another example of an assay for TNF delta or TNF epsilon antagonists is a competitive assay that combines TNF delta or TNF epsilon and a potential antagonist with membrane-bound TNF delta or TNF epsilon receptor molecules or recombinant TNF delta or TNF epsilon receptor molecules under appropriate conditions for a competitive inhibition assay. TNF delta or TNF epsilon can be labeled, such as by radioactivity, such that the number of TNF delta or TNF epsilon molecules bound to a receptor molecule can be determined accurately to assess the effectiveness of the potential antagonist.

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a receptor molecule, without inducing TNF delta or TNF epsilon-induced activities, thereby preventing the action of a polypeptide of the present invention by excluding it from binding to its receptor.

Another potential antagonist is a soluble form of the TNF delta or TNF epsilon receptor which binds to TNF delta or TNF epsilon and prevents it from interacting with membrane-bound TNF delta or TNF epsilon receptors. In this way, the receptors are not stimulated by their ligand.

Potential antagonists include a small molecule which binds to and occupies the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such as receptor molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or non-peptide antagonists.

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through

triple-helix formation. Antisense techniques are discussed, for example, in Okano, J. *Neurochem.*, 56:560, 1991; Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research*, 6:3073 (1979); Cooney *et al.*, *Science*, 241:456 (1988); and Dervan *et al.*, *Science*, 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA. For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of TNF delta or TNF epsilon. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into TNF delta or TNF epsilon polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of a polypeptide of the present invention.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, *e.g.*, as hereinafter described.

The antagonists may be employed for instance to treat cachexia which is a lipid clearing defect resulting from a systemic deficiency of lipoprotein lipase, which is suppressed by TNF delta or TNF epsilon. The antagonists may also be employed to treat cerebral malaria in which polypeptides of the present invention appear to play a pathogenic role. The antagonists may also be employed to treat rheumatoid arthritis by inhibiting TNF delta or TNF epsilon induced production of inflammatory cytokines, such as IL1 in the synovial cells. When treating arthritis, the polypeptides of the present invention are preferably injected intra-articularly.

The antagonists may also be employed to prevent graft-host rejection by preventing the stimulation of the immune system in the presence of a graft.

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The antagonists may also be employed to inhibit bone resorption and, therefore, to treat and/or prevent osteoporosis.

The antagonists may also be employed as anti-inflammatory agents, and to treat endotoxic shock. This critical condition results from an exaggerated response to bacterial and other types of infection.

The invention also relates to compositions comprising the polynucleotide or the polypeptides discussed above or the agonists or antagonists. Thus, the polypeptides of the present invention may be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of a polypeptide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. The formulation should suit the mode of administration.

The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, reflecting approval by the agency of the manufacture, use or sale of the product for human administration.

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal,

intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 10 $\mu\text{g/kg}$ body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about 10 $\mu\text{g/kg}$ to about 1 mg/kg body weight, daily. It will be appreciated that optimum dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like.

The polynucleotides, polypeptides, agonists and antagonists that are polypeptides of this invention may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, in treatment modalities often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide, such as a DNA or RNA, encoding a polypeptide *ex vivo*, and the engineered cells then can be provided to a patient to be treated with the polypeptide. For example, cells may be engineered *ex vivo* by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention. Such methods are well-known in the art and their use in the present invention will be apparent from the teachings herein.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by procedures known in the art. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct then may be isolated and introduced into a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding

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a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors herein above mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Such vectors well include one or more promoters for expressing the polypeptide. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al.*, *Biotechniques*, 7: 980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, RNA polymerase III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention will be placed under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin

promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs herein above described); the β -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, A., *Human Gene Therapy*, 1: 5-14 (1990). The vector may be transduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO₄ precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line will generate infectious retroviral vector particles, which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplification's, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

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All examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. Routine molecular biology techniques of the following examples can be carried out as described in standard laboratory manuals, such as Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), herein referred to as "Sambrook."

All parts or amounts set out in the following examples are by weight, unless otherwise specified. Unless otherwise stated size separation of fragments in the examples below was carried out using standard techniques of agarose and polyacrylamide gel electrophoresis ("PAGE") in Sambrook and numerous other references such as, for instance, by Goeddel *et al.*, *Nucleic Acids Res.*, 8: 4057 (1980). Unless described otherwise, ligations were accomplished using standard buffers, incubation temperatures and times, approximately equimolar amounts of the DNA fragments to be ligated and approximately 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of DNA.

Example 1

Expression and Purification of Soluble Form of Human TNF Delta and TNF Epsilon Using Bacteria

The DNA sequence encoding human TNF delta or TNF epsilon in the deposited polynucleotide was amplified using PCR oligonucleotide primers specific to the amino acid carboxyl terminal sequence of the human TNF delta or TNF epsilon protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning were added to the 5' and 3' sequences respectively.

The 5' oligonucleotide primer had the sequence 5' GCG GGA TCC CAG AGC CTC ACC ACA G 3' containing the underlined restriction site, followed by 16

nucleotides of coding sequence set out in the Figures beginning with the 115th base of the ATG codon.

The 3' primer has the sequence 5' CGC AAG CTT ACA ATC ACA GTT TCA CAA AC 3' contains the underlined HindIII restriction site followed by 20 nucleotides complementary to the last 13 nucleotides of the coding sequence set out in Figures 1 and 2, including the stop codon.

The restrictions sites were convenient to restriction enzyme sites in the bacterial expression vectors pQE-9, which were used for bacterial expression in these examples. (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), a 6-His tag and restriction enzyme sites.

The amplified human TNF delta DNA and the vector pQE-9 both were digested with BamHI and HindIII and the digested DNAs then were ligated together. Insertion of the TNF delta DNA into the pQE-9 restricted vector placed the TNF delta coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of TNF delta.

The ligation mixture was transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan^r"), was used in carrying out the illustrative example described here. This strain, which is only one of many that are suitable for expressing TNF delta, is available commercially from Qiagen. Transformants were identified by their ability to grow on LB plates in the presence of ampicillin. Plasmid DNA was isolated from resistant colonies and the identity of the cloned DNA was confirmed by restriction analysis.

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Clones containing the desired constructs were grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 ug/ml) and kanamycin (25 μ g/ml). The O/N culture was used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells were grown to an optical density at 600nm ("OD₆₀₀") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") was then added to a final concentration of 1 mM to induce transcription from lac repressor sensitive promoters, by inactivating the lacI repressor. Cells subsequently were incubated further for 3 to 4 hours. Cells then were harvested by centrifugation and disrupted, by standard methods. Inclusion bodies were purified from the disrupted cells using routine collection techniques, and protein was solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein was passed over a PD-10 column in 2X phosphate buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein was purified by a further step of chromatography to remove endotoxin. Then, it was sterile filtered. The sterile filtered protein preparation was stored in 2X PBS at a concentration of 95 micrograms per mL.

Analysis of the preparation of TNF delta by standard methods of polyacrylamide gel electrophoresis revealed that the preparation contained about 80% monomer having the expected molecular weight of, approximately, 20.8 kDa.

The protein is purified by chromatography on a nickel-chelate column under conditions that allow for type-binding by proteins containing the 6-HIS tag. The protein is eluted from the column in 6-molar guanidine HCl pH 5.0 and renatured.

Example 2

Cloning and Expression of Soluble Human TNF Delta and TNF Epsilon in a Baculovirus Expression System

The cDNA sequence encoding the full length human TNF delta or TNF epsilon protein, in the deposited clone is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCG GGA TCC CCA GAG CCT CAC CAC AG 3' containing the underlined BamHI restriction enzyme site followed by 16 bases of the sequence of TNF delta or TNF epsilon of Figures 1 and 2. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding human TNF delta or TNF epsilon provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., J. Mol. Biol. 196: 947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer has the sequence 5' CGC TCT AGA ACA ATC ACA GTT TCA CAA AC 3' containing the underlined XbaI restriction site followed by nucleotides complementary to the last 13 nucleotides of the TNF delta or TNF epsilon coding sequence set out in Figures 1 and 2, including the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("GeneClean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated herein F2.

The vector pA2GP is used to express the TNF delta or TNF epsilon protein in the baculovirus expression system, using standard methods, such as those described in Summers et al, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites. The signal peptide of AcMNPV gp67, including the N-terminal methionine, is located just

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upstream of a BamHI site. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter and is followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2-GP, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription, translation, trafficking and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al.*, *Virology*, 170:31-39, among others.

The plasmid is digested with the restriction enzymes BamHI and XbaI and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V2".

Fragment F2 and the dephosphorylated plasmid V2 are ligated together with T4 DNA ligase. *E.coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human TNF delta or TNF epsilon gene by digesting DNA from individual colonies using BamHI and XbaI and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBacTNF delta.

5 μ g of the plasmid pBacTNF delta is co-transfected with 1.0 μ g of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA",

Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987). 1 μ g of BaculoGold™ virus DNA and 5 μ g of the plasmid pBacTNF delta are mixed in a sterile well of a microtiter plate containing 50 μ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 μ l Lipofectin plus 90 μ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 μ l of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. A clone containing properly inserted TNF delta or TNF epsilon is identified by DNA or TNF epsilon analysis including restriction mapping and sequencing. This is designated herein as V-TNF delta.

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Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-TNF delta at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg). 42 hours later, 5 μ Ci of 35S-methionine and 5 μ Ci 35S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

Example 3

Tissue Distribution of TNF Delta Expression

Northern blot analysis was carried out to examine the levels of expression of TNF delta in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. Total cellular RNA samples are isolated with RNazol™ B system (Biotech Laboratories, Inc. 6023 South Loop East, Houston, TX 77033).

About 10 μ g of Total RNA was isolated from tissue samples. The RNA was size resolved by electrophoresis through a 1% agarose gel under strongly denaturing conditions. RNA was blotted from the gel onto a nylon filter, and the filter then is prepared for hybridization to a detectably labeled polynucleotide probe.

As a probe to detect mRNA that encodes TNF delta, the antisense strand of the coding region of the cDNA insert in the deposited clone was labeled to a high specific activity. The cDNA was labeled by primer extension, using the Prime-It kit, available from Stratagene. The reaction was carried out using 50 ng of the cDNA, following the standard reaction protocol as recommended by the supplier. The labeled polynucleotide was purified away from other labeled reaction components by column chromatography using a Select-G-50 column, obtained from 5-Prime - 3-Prime, Inc. of 5603 Arapahoe Road, Boulder, CO 80303.

The labeled probe was hybridized to the filter, at a concentration of 1,000,000 cpm/ml, in a small volume of 7% SDS, 0.5 M NaPO₄, pH 7.4 at 65°C, overnight.

Thereafter the probe solution was drained and the filter is washed twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS. The filter then is dried and exposed to film at -70°C overnight with an intensifying screen.

Autoradiography shows that mRNA for TNF delta was detected in all 16 tissues with highest expression in heart followed by placenta and kidney.

Example 4

Gene Therapeutic Expression of Human TNF Delta or TNF Epsilon

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature overnight. After 24 hours at room temperature, the flask is inverted - the chunks of tissue remain fixed to the bottom of the flask - and fresh media is added (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin). The tissue is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerges. The monolayer is trypsinized and scaled into larger flasks.

A vector for gene therapy is digested with restriction enzymes for cloning a fragment to be expressed. The digested vector is treated with calf intestinal phosphatase to prevent self-ligation. The dephosphorylated, linear vector is fractionated on an agarose gel and purified.

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cDNA capable of expressing active TNF delta or TNF epsilon, is isolated. The ends of the fragment are modified, if necessary, for cloning into the vector. For instance, 5' overhanging may be treated with DNA polymerase to create blunt ends. 3' overhanging ends may be removed using S1 nuclease. Linkers may be ligated to blunt ends with T4 DNA ligase.

Equal quantities of the Moloney murine leukemia virus linear backbone and the TNF delta or TNF epsilon fragment are mixed together and joined using T4 DNA ligase. The ligation mixture is used to transform E. Coli and the bacteria are then plated onto agar-containing kanamycin. Kanamycin phenotype and restriction analysis confirm that the vector has the properly inserted gene.

Packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The vector containing the TNF delta or TNF epsilon gene is introduced into the packaging cells by standard techniques. Infectious viral particles containing the TNF delta or TNF epsilon gene are collected from the packaging cells, which now are called producer cells.

Fresh media is added to the producer cells, and after an appropriate incubation period media is harvested from the plates of confluent producer cells. The media, containing the infectious viral particles, is filtered through a Millipore filter to remove detached producer cells. The filtered media then is used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the filtered media. Polybrene (Aldrich) may be included in the media to facilitate transduction. After appropriate incubation, the media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his, to select out transduced cells for expansion.

Engineered fibroblasts then may be injected into rats, either alone or after having been grown to confluence on microcarrier beads, such as cytodex 3 beads. The injected fibroblasts produce TNF delta or TNF epsilon product, and the biological actions of the protein are conveyed to the host.

~~It will be clear that the invention may be practiced otherwise than as particularly~~
described in the foregoing description and examples. Numerous modifications and
variations of the present invention are possible in light of the above teachings and,
~~therefore, are within the scope of the appended claims.~~

EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

Druckexemplar

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polypeptide encoded by a polynucleotide of any one of (a) to (d) wherein said fragment is capable of stimulating an immune response; and
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence.
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response;
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

4. The polynucleotide of any one of claims 1 to 3 which is fused to a heterologous polynucleotide.
5. A vector containing the polynucleotide of any one of claims 1 to 4.
6. The vector of claim 5 in which the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
7. A host cell genetically engineered with the polynucleotide of any one of claims 1 to 4 or the vector of claim 5 or 6.
8. A process for producing a polypeptide capable of stimulating an immune response comprising: culturing the host cell of claim 7 and recovering the polypeptide encoded by said polynucleotide from the culture.
9. A process for producing cells capable of expressing a polypeptide which is capable of stimulating an immune response comprising genetically engineering cells with the vector of claim 5 or 6.
10. A polypeptide having the amino acid sequence encoded by a polynucleotide of any one of claims 1 to 4 or obtainable by the process of claim 8.
11. An antibody specific for the polypeptide of claim 10.
12. A nucleic acid molecule which specifically hybridizes to a polynucleotide of any one of claims 1 to 4.
13. An antagonist/inhibitor of the polypeptide of claim 10, wherein said antagonist/inhibitor is an antibody of claim 11 capable of inhibiting or extinguishing the activity of the polypeptide of claim 10 or a nucleic acid molecule of claim 12 capable of binding and thereby inhibiting the expression of the polynucleotide or the DNA of any one of claims 1 to 4.

14. A pharmaceutical composition comprising the polynucleotide of any one of claims 1 to 4, the polypeptide of claim 10 or a DNA encoding and capable of expressing said polypeptide in vivo or the antagonist/inhibitor of claim 13 and optionally a pharmaceutically acceptable carrier.
15. A diagnostic composition comprising the polynucleotide of any one of claims 1 to 4, the nucleic acid molecule of claim 12 or the antibody of claim 11.
16. Use of the polypeptide of claim 10 or the polynucleotide of any one of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment of neoplasia, for wound-healing, for the treatment of restenosis, for regulating hematopoiesis in endothelial cell development, for stimulating an immune response against parasitic, bacterial or viral infections, or for the treatment and/or prevention of autoimmune diseases.
17. Use of the antagonist/inhibitor of claim 13 for the preparation of a pharmaceutical composition for the treatment of cachexia, cerebral malaria, rheumatoid arthritis, for the prevention of graft-host rejection, for inhibiting bone resorption, for the treatment and/or prevention of osteoporosis, or for the treatment of endotoxic shock.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 10 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 10 in a sample derived from a host.
20. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 10 comprising:
 - (a) contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF

delta polypeptide and a compound under conditions to permit binding to the receptor; and

- (b) determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.

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FIG. 1A

1 ACCTCTGTCCTAGAGGGGACTGGAACCTAAATTCTCTCCTGAGCCCTGAGGGAGGGTGGAGGG 60
 61 TCTCAAGACAAACGCTGTCCCCACGACGGAGTGCCAGGAGCACTAACAGTACCCCTTAGATT 120
 121 GCTTTCCTCCTCCCTCCTTTTATTTTCAAGTTCTTTTATTTCTCCTTGGTAACAA 180
 181 CCTTCTTCCCTTCTGCACCACTGCCCGTACCCCTTACCCGGCCGCCACCTCCTTGCTACA 240
 241 CCACTCTTGAAACCAACAGCTGTTGGCAGGTCCCCCAGCTCATGCCAGCCCTCATCTCCTT 300
 301 TCTTGCTAGCCCCCAAGGCCCTCCAGGCAACATGGGGGCCCAAGTCAAGAGAGCCGGCAC 360
 M G G P V R E P A L
 361 TCTCAGTTCCTCTGTTGAGTTGGGGGCGAGCTCTGGGGCCGCTTGCTTGCCCATGG 420
 S V A L W L S W G A A L G A V A C A M A
 421 CTCTGCTGACCCAAACAGAGCTGCAGAGCCCTGAGGAGAGAGGTGAGCCGGCTGCAGA 480
 L L T Q Q T E L Q S L R R E V S R L Q R
 481 GGACAGGAGGCCCTCCAGAAATGGGGAAGGTATCCCTGGCAGAGTCTCCCGGAGCAGA 540
 T G G P S Q N G E G Y P W Q S L P E Q S
 541 GTTCCGATGCCCTGGAAACCTGGGAGAAATGGGGAGAGATCCCGGAAAGGAGAGCAGTGC 600
 S D A L E A W E N G E R S R K R R A V L
 601 TCAGCCAAAACAGAAAGCAGCAGCTCTGTCTGACCTGTTCCCATTAACGCCACCT 660
 T Q K Q K Q H S V L H L V P I N A T S
 661 CCAAGGATGACTCCGATGTGACAGAGGTGATGTGGCAACCAGCTCTTAGCGTGGGAGAG 720
 K D D S D V T E V M W Q P A L R R G R G

MATCH WITH FIG. 1B

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MATCH WITH FIG. 1A

721	GCCTACAGGCCCAAGGATATGCTGCTCCGAATCCAGGATGCTGGAGTTTATCTGCTGTATA	780
	L Q A Q G Y G V R I Q D A G V Y L L Y S	
781	GCCAGGTCCTGTTCAAGACCTGACTTTCACCATGGGTCAGGTGCTGCTCGAGAAGGCC	840
	Q V L F Q D V T F T M G Q V V S R E G Q	
841	AAGGAAGCCAGGAGACTCTATTCCGATGTATAAGAAGTATGCCCTCCACCCGACCGGG	900
	G R Q E T L F R C I R S M P S H P D R A	
901	CCTACAACAGCTCCTATAGCGCAGGTGCTTTCATTACACCAAGGGGATATTCTGAGTG	960
	Y N S C Y S A C V F H L H Q G D I L S V	
961	TCATAATTCCCCCGGCAAGGGCGAAACTTAACCTCTCTCCACATGGAACCTTCTCTGGGGT	1020
	I I P P A R A K L N L S P H G T F L G F	
1021	TTGTGAAACTGTCA ^V TTGTGTATAAAAGTGGCTCCAGCTTCCAGACACGAGGTGGGTA	1080
	^{K L}	
1081	CATACTGGAGACGCCAAGAGCTGACTATATAAGGAGAGGGAATGTCAGGAACAGAGG	1140
1141	CGTCTTCTGGGTTTGGCTCCCGCTTCTCTCACTTTTCCCTTTTCAATCCACACCCCTAGA	1200
1201	CTTTGATTTTACGGATATCTTGTCTTCTTCTTCCCATGGAGCTCCGAATCTTGGCTGTGT	1260
1261	GTAGATGAGGGCGGGGACGGCCCGCCAGCCATTCTCCAGACCTGGTCGGGGCCCACTCG	1320
1261	AAGCATCCAGAACAGCACCACTACCGGGCGCTCTAGAGGATCCCTCGAGGGGCCCA	1380
1381	AGCTTACGGCTGCGATGGAGCTCATAGCTCTCTCCCTATAGTGAGTCGTATTATAAGCTA	1440
1441	GCTTGGGATCTTTGTGAAGGAACCTTACTTCTGTGGTGTGACATAATTGGACAACTACC	1500
1501	TACAGAGATTTAAAGCTCTAAGCTAAATATAAAATTTTAAAGTGTATAATGTGTTAACT	1560
1561	AGCTGCATATGCTTCTGCTTTCAGAGCTTTGGCTTACTGAGTATGATTATGAAAAATATAT	1620
1621	ACACAGGAGCTAGTGATCTATGTTGCTTTTAGATCAAGCCCAAGGTCATTTCAGGCCCTCAGC	1680
1681	TCAAGCTGTCATGATCATATCAGCATACAAATTGTGAG	1717

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FIG. 2A

GGGACAGGAGGCCCTCCAGAAATGGGAAGGTATCCCTGGCAGAGTCTCCCGAGCA
G T G G P S Q N G E G Y P W Q S L P E Q

GAGTCCGATGCCCTGGAAGCCTGGAGAGTGGGAGAGATCCCGAAAGGAGAGCAGT
S S D A L E A W E S G E R S R K R R A V

GCTACCCAAACAGAAATGACTCCGATGTGACAGAGGTGATGTGGCAACCAGCTCT
L T Q K Q K N D S D V T E V M W Q P A L

TAGCGTGGAGAGGCCCTACAGGCCCAAGGATATGGTCCGAATCCAGGATGCTGGAGT
R R G R G L Q A Q G Y G V R I Q D A G V

TTATCTCCTGTATAGCCAGGTCCTGTTCAGACGTGACTTTCACCATGGGTCAGGTGGT
Y L L Y S Q V L F Q D V T F T M G Q V V

GTCTCGAAGGCCAAGGAAGGCAGGAGACTCTATTCGGATGTATAAGAAGTATGCCCTC
S R E G Q G R Q E T L F R C I R S M P S

CCACCCGACCGGCCCTACAACAGCTGCTATAGCGCAGGTGCTTCCATTACACCAAGG
H P D R A Y N S C Y S A G V F H L H Q G

MATCH WITH FIG. 2B

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MATCH WITH FIG. 2A FIG. 2B

GGATATCTGAGTGTCATAATTCCCCGGCAAGGCGAACTTAACCTCTCTCCACATGG
D I L S V I I P R A R A K L N L S P H G

AACCTTCTGGGTTTGTGAACCTGTGATTGTGTTATATAAAAGTGGCTCCAGCTTGGAA
T F L G F V K L

GACCAGGTGGGTACATACTGGAGACAGCCAGAGCTGAGTATATAAAGAGAGGGAATG
TGCAGGAACAGAGCGTCTTCCCTGGGTTTGGCTCCCGTTCCCTCACTTTTCCCTTTTCAT
TCCCACCCCTAGACTTTGGATTTACGGATATCTTGCTTCTGTTCCTCCATGGAGCTCCG
AATTCTTGCGTGTGTAGATGAGGGCGGGGACGGCGCCAGGCATTTGTCAGACCTG
GTCGGGGCCCACTGGAAGCATCCAGAACAGCACCATCTAGCGGCGCTCGAGGGAAGC
ACCGCGGTTGGCCGAAGTCCACGAAGCCGCTCTGCTAGGGAAACCCCTGGTTCCTCCAT
GCCACAACTCTCTCCAGGTGGCTCTGCTTCAACCCACAAAGAGCCCTTAACCTA
CGTCCCTTCTCCATCTATCGGACCCAGTTTCCATCACTATCTCCAGAGATGTAGCTAT
TATGCGCCCGCTACAGGGGTGCCCGACGATGACGGTGCTTCGCGAGTCAATTACTCT
TCGGGTCCCAAGTTTGGCTTTACGGCTCCATTGCCCCGGCGTGCGAGGCCATTCCAA
GCCCTTCCGGCTGGAACGTGTGTCGGAGAGCCCTCGGGTGTATCGTACGCCCTGGTGT
GGTGTGCTCACTCCTCTGAGCTCTTCTTCTGATCAAGCCCTGCTTAAAGTTAAATAA
AATAGAAATGATATAAAATAAAATAAAATAAAATAAAATAAAATAAAATAAAATAA

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FIG. 3A

```

1  MSTESNIRDVELAEELPKKTGGPOGSRRCLFLSLFSE
1  MTPPERLF-----LPRVCGTT-----LHLILGL
1  MGGPVPREPALSV---ALWLSWGAALCAVACAMALLTQOT
1  G-----

70  ISPLAQAV-----RSSSRT-----PSDK-----PVAHVVA
41  TPSAACTA-----RQHPKMHLAHSTLK-----PAAHLIG
67  PEQSSDALEAWENGERSRKRRRAVLTQKQKKQHSMVHLVLP
18  PEQSSDALEAWESGERSRKRRRAVLTQKQK-----

118 ELRDNLQLVVPSEGLYLIYSQVLFKGQGC-----PSTHVLL
93  SLNNLSLIVPTSGIYFVYSQVVFSGKAYSPKAPSSPLYL
137 GVR-----IQDAGVYLIYSQVLFQDVTFT-----M
72  GVR-----IQDAGVYLIYSQVLFQDVTFT-----M

184 GAELKPWYEPIYLGCVFQLEKGDRLSAEINRPDYLDFAE
158 -GLQEPWLHSMYHGAAFQLTQGDQLSTHTDGIPHVLVS-
186 HPDRA--YNSCYSGGVFHLHQGDILSVIIPRARAKLNLS
121 HPDRA--YNSCYSGGVFHLHQGDILSVIIPRARAKLNLS

```

Decoration '': Shade (with solid black)
residues that match TNFalpha exactly.

Match with FIG. 3B

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FIG. 3B

LIVAGATTLEFCLLHFGVIGPOREESPRDLSL	TNFalpha
LLV-----LI-----PGAQGLF-GVGI	TNFbeta
ELQSLRREVSRLQRTGGPSQNGEGYPWQ-SL	TNFdelta
-----TGGPSQNGEGYPWQ-SL	TNFepsilon
-----NPOAEGQLOWLN--RRANALLANGV	TNFalpha
-----DPSKQNSLLWRA--NTDRAFLQDGF	TNFbeta
INATSKDDSDVTEVMWQPALRRGRGLQACGY	TNFdelta
-----NDSDVTEVMWQPALRRGRGLQACGY	TNFepsilon
THTISRIAVSYQTKVNLLSAIKSPCQRETPE	TNFalpha
AHEVQLFSSQYPFHVPLLSSQKMVYP-----	TNFbeta
GQVVSREGQG--RQETLFR-----CIRSMPS	TNFdelta
GQVVSREGQG--RQETLFR-----CIRSMPS	TNFepsilon
SGQVYFGIIAI	TNFalpha
PSTVFECAFAL	TNFbeta
PHGTFLGFVK-L	TNFdelta
PHGTFLGFVKI.	TNFepsilon

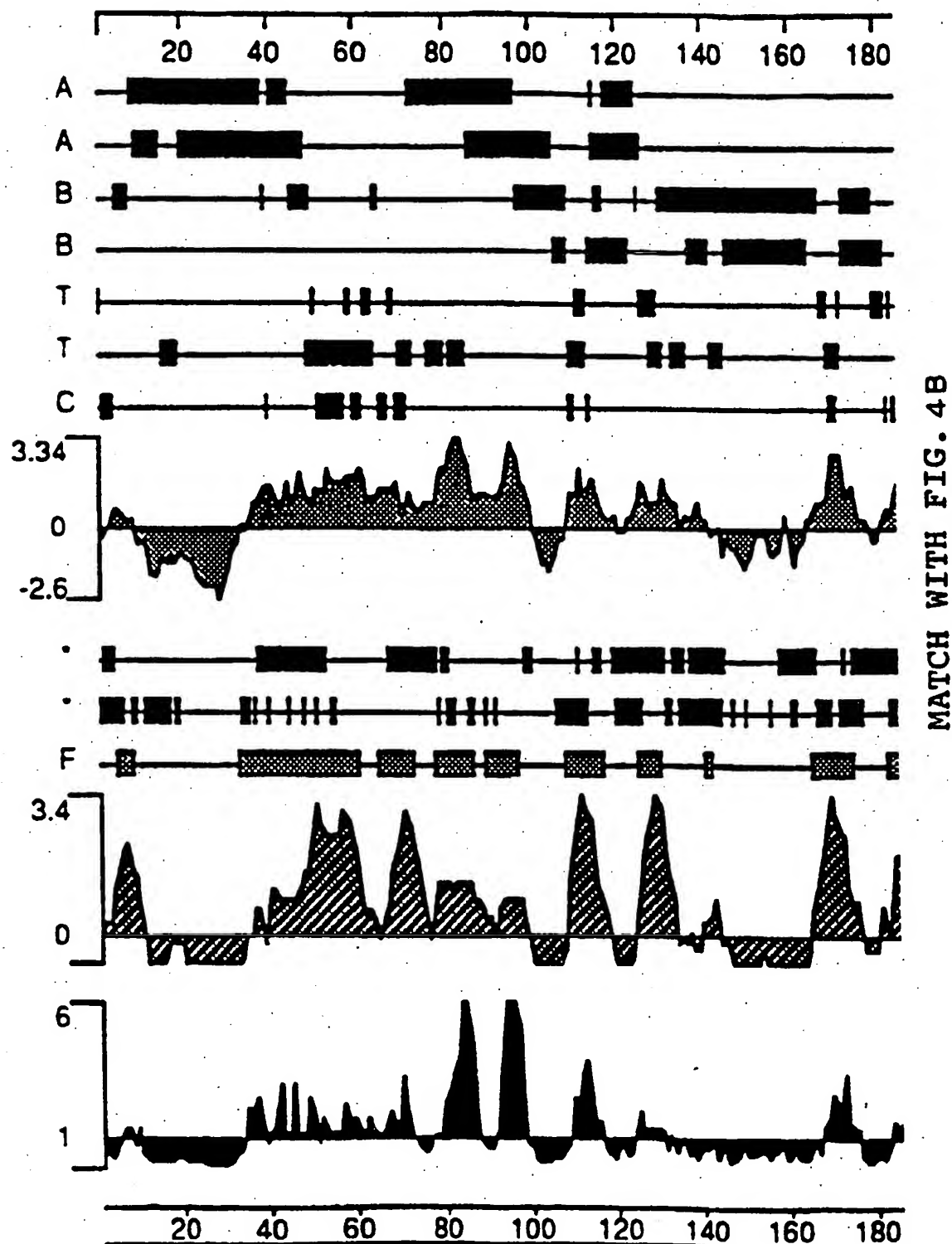
MATCH WITH FIG. 3A

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FIG. 4A

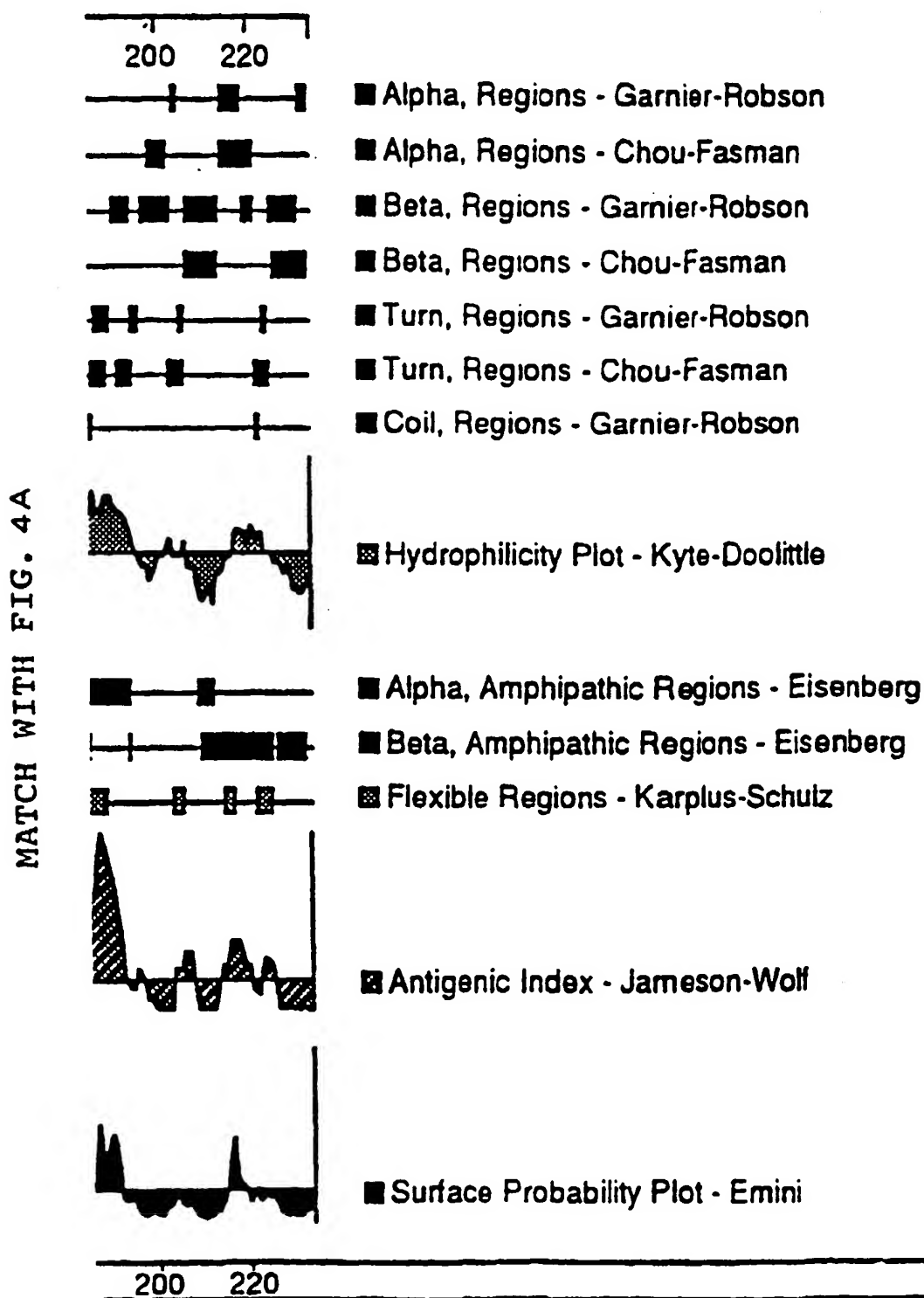


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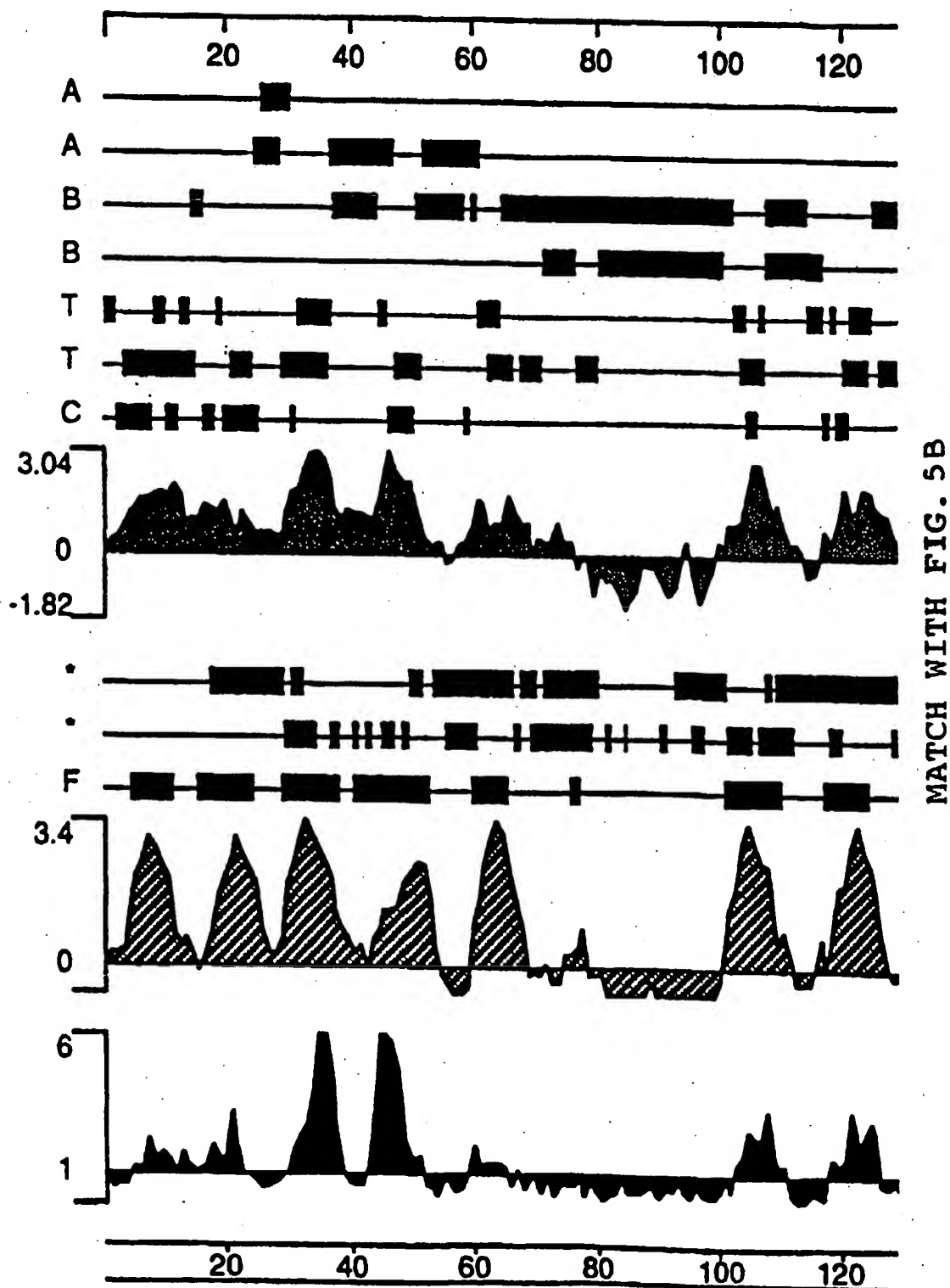
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FIG. 4B



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FIG. 5A

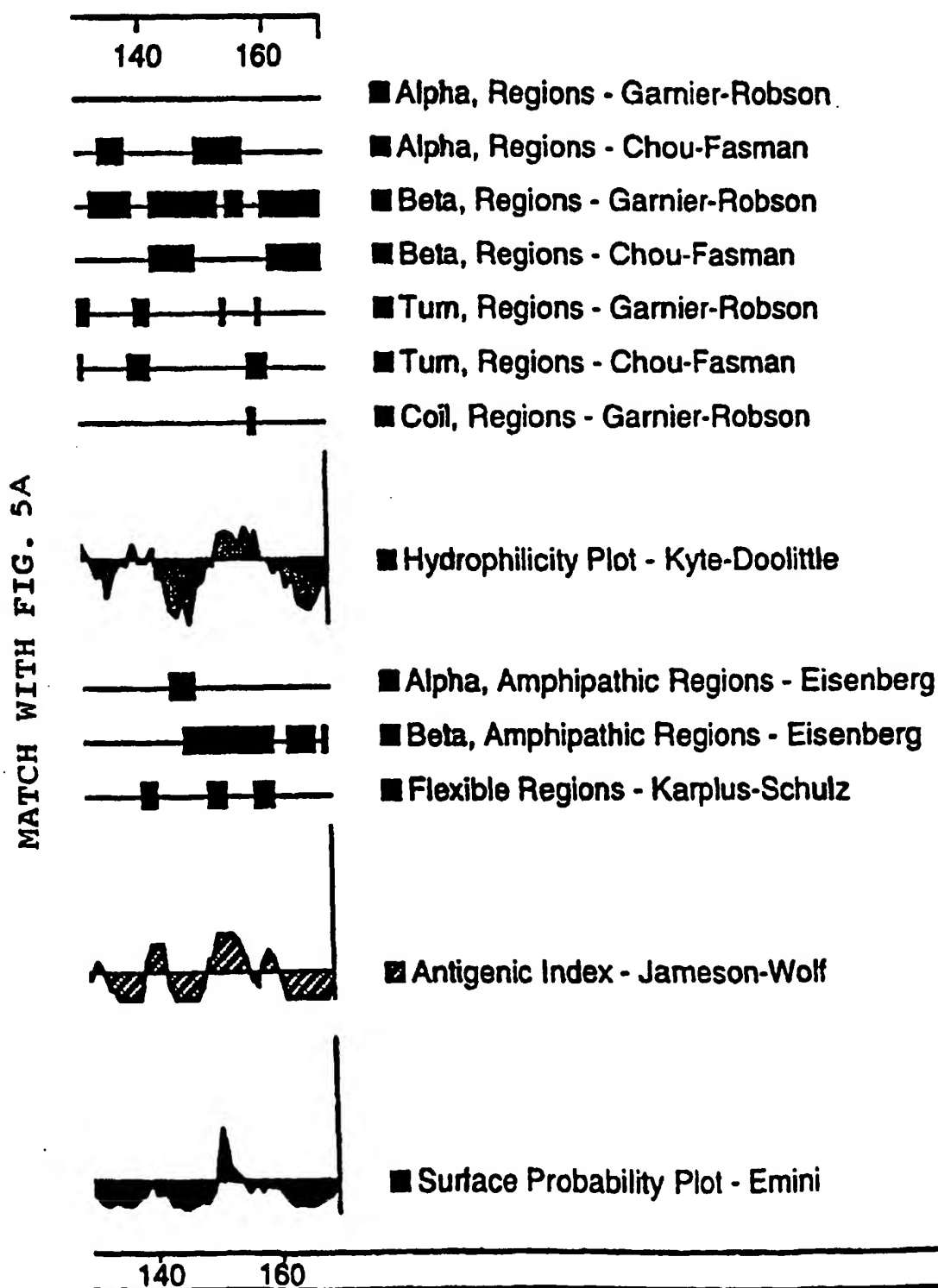


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FIG. 5B





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81634 München
ALLEMAGNE



Application No. 96 910 483.5-2110	Ref. C 2276 EP	Date 17.02.2003
Applicant HUMAN GENOME SCIENCES, INC.		

Communication under Rule 51(4) EPC

You are informed that the Examining Division intends to grant a European patent on the basis of the above application with the text and drawings as indicated below:

Text for the Contracting States:

AT BE CH LI DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Description, pages:

1,3-7,9-63	as published		
2,8,64	as received on	19.12.2002	with letter of 18.12.2002

Claims, No.:

1-20	as received on	19.12.2002	with letter of 18.12.2002
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Drawings, sheets:

1/10,3/10-10/10	as published		
2/10	as received on	18.02.2002	with letter of 14.02.2002

A copy of the relevant documents is enclosed.

The title of the invention in the three official languages of the European Patent Office, the international patent classification, the designated Contracting States, the registered name of the applicant and the bibliographic data are shown on the attached EPO Form 2056.

You are requested **within four months** of this notification



1.	to file 1 set of translations of the claim(s) in the two other EPO official languages;		EUR
2a.	to pay the fee for grant including the fee for printing up to and including 35 pages;		
	Reference 007		715.00
2b.	to pay the printing fee for the 36th and each additional page;		
	number of pages: 43		
	Reference 008		430.00
3.	to pay the additional claim fee(s) (Rule 51(7) EPC);		
	number of claims fees payable: 0		
	Reference 016		0.00
	Total amount		1145.00

Concerning the possibility of a request for accelerated grant pursuant to Article 97(6) EPC, reference is made to OJ EPO 2001, 459.

If the grant, printing or claims fees are not paid, or the translations not filed, in due time the European patent application will be deemed to be withdrawn (Rule 51(8) EPC).

For all payments you are requested to use EPO Form 1010 or to refer to the relevant reference number.

If additional copies of the patent specification are required, you should request this in writing and quote Fee reference code 058 when making payment.

Translation of the priority document(s)

If the translation of the priority document(s), as required by Article 88(1) EPC, or the declaration according to Rule 38(5) EPC has not yet been filed, Form 2530 will be despatched separately. The translation is to be filed within the above mentioned time limit (Rule 38(5) EPC).

Note on payment of renewal fees

If a renewal fee falls due between notification of the present communication and the proposed date of publication of the mention of the grant of the European patent, publication will be effected only after the renewal fee and any additional fee have been paid (Rule 51(9) EPC).

Under Article 86(4) EPC, renewal fees are payable to the European Patent Office until the year in which the mention of the grant of the European patent is published.

Filing of translations in the Contracting States

Pursuant to Article 65(1) EPC the following Contracting States require a translation of the specification of the European patent in their/one of their official language(s) (Rule 51(10) EPC), **insofar** this specification will not be published in their/one of their official language(s)

- within **three** months of publication of the mention of such decision:

AT	AUSTRIA	FR	FRANCE
BE	BELGIUM	GB	UNITED KINGDOM
CH	SWITZERLAND / LIECHTENSTEIN	GR	GREECE
CY	CYPRUS	IT	ITALY
DE	GERMANY	NL	NETHERLANDS



DK DENMARK
ES SPAIN
FI FINLAND

PT PORTUGAL
SE SWEDEN
TR TURKEY

- within **six** months of publication of the mention of such decision:

IE IRELAND

The date on which the European Patent Bulletin publishes the mention of the grant of the European patent will be indicated in the decision on the grant of the European patent (EPO Form 2006).

In case of a valid extension the following Extension States require a translation of the **claims** in their official language within **three** months after publication of the mention of the grant of the European patent:

AL ALBANIA
LT LITHUANIA
LV LATVIA
MK MACEDONIA

RO ROMANIA (requires translation
of the specification)
SI SLOVENIA

The translation must be filed with the national Patent Offices of the Contracting or Extension States in accordance with the provisions applying thereto in the State concerned. Further details (e.g. appointment of a national representative or indication of an address for service within the country) are given in the EPO information brochure "National law relating to the EPC", and in the supplementary information published in the Official Journal of the EPO, or available on the EPO website.

Failure to supply such translation to the Contracting and Extension States in time and in accordance with the requirements may result in the patent being deemed to be void ab initio in the State concerned.

Note to users of the automatic debiting procedure

Unless the EPO receives prior instructions to the contrary, the fee(s) will be debited on the last day of the period of payment. For further details see the Arrangements for the automatic debiting procedure (see Supplement to OJ EPO 2, 2002).



Date 17.02.2003

Sheet 4

Application-No.: 96 910 483.5

Examining Division:

Chairman: BARDILI W B
2nd Examiner: HENNARD C
1st Examiner: DEFFNER C A E



Ladurner, Y
For the Examining Division
Tel. No.: +49 89 2399-7913

Enclosure(s): Form 2056
78 Copies of the relevant documents

Annex to EPO Form 2004, Communication under Rule 51(4) EPC

Bibliographical data of European patent application No. 96 910 483.5

For the intended grant of a European patent, the bibliographical data are set out below, for information:

Title of invention:

- HUMANER TUMORNEKROSEFAKTOR DELTA UND EPSILON
- HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON
- FACTEURS DE NECROSE TUMORALE DELTA ET EPSILON CHEZ L'HOMME

Classification: C07H21/02, C07H21/04, C07K1/00, C07K14/00, C12N5/00, C12N15/09, C12N15/63, C07K14/525

Date of filing: 14.03.1996

Priority claimed:

Contracting States*
for which fees have
been paid:

AT BE CH LI DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Extension States*
for which fees have
been paid:

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US

*) In case the time limits pursuant to Article 79(2) and Rule 85a EPC have not yet expired, all **Contracting States/Extension States** have been mentioned.
**) In case two or more applicants have designated different Contracting States, this is indicated here.

19. Dez. 2002

VOSSIUS & PARTNER

Patentanwälte

Vossius & Partner POB 86 07 67 81634 Munich Germany

European Patent Office
Erhardtstr. 27

80298 MÜNCHEN

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96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C2276 EP

December 18, 2002
JAE/AD/ONG

This is response to the Communication pursuant to Art. 96(2) EPC issued by the EPO on August 23, 2002 in the above referenced case.

Please find enclosed new claims 1 to 20 and new pages 2, 8 and 64.

1. Amendments

1.1 Amendments to the claims

1.1.1 New claim 1

New claim 1 is based on previous claim 1 except that the fragments encompassed by alternative (f) are further defined as being capable of stimulating an immune response. Support can be found on page 46, third paragraph in connection with page 24, third paragraph and page 26, third paragraph.

1.1.2 New claims 2 to 20

New claims 2 to 20 correspond to previous claims 2 to 20.

1.2 Amendments to the description

1.2.1 New page 2

On new page 2, the ESTs disclosed in Adams et al. and Seed et al. have been included in the description of the state of the art.

1.2.2 New page 8

Lines 3 to 6 of previous page 8 have been deleted on new page 8.

1.2.3 New page 64

The last phrase on previous page 64 has been deleted on new page 64.

2. **Novelty (Art. 54 EPC) of previous and new claim 1**

The Examining Division took the position that previous claim 1 lacked novelty over the ESTs of Adams et al. (M78230) and Seed et al. (N90606).

An alignment of the EST sequence disclosed in M78230 with the sequence in Figure 1 (SEQ ID NO: 1) is enclosed. Most of the region that aligns between the EST and the sequence in Figure 1 (SEQ ID NO.:1) falls in for the non-coding region and as such does not fall within the scope of claim 1. Nucleotides 7-113 of the M78230 EST align to the end of the coding region of the sequence in Figure 1 (SEQ ID NO.:1). The "A" nucleotide at position 26 of the M78230 EST would cause a frameshift in any protein encoded by the M78230 nucleotide sequence. Thus, the relevant region of the M78230 EST is nucleotides 21-113. When translated, this region encodes GDILSVITPRTRXKLNLSPHGTFLGFVKL which contains an "X" amino acid and is only 29 amino acids of the amino acid sequence shown in Figure 1 (SEQ ID NO.:2). Therefore, the M78230 does not fall within the scope of claim 1.

The sequence disclosed in Geneseq Accession No. N90606 has a stretch of 116 base pairs that corresponds to nucleotides 1446-1561 of the sequence in Figure 1 (SEQ ID NO.:1). This region, however, lies outside the TNF-delta coding region, which ends at

nucleotide 1031 (exclusive of the stop codon spanning nucleotides 1031-1034) and therefore does not fall within the scope of claim 1.

Therefore, new claim 1 is novel over EST M78230 by Adams et al. and EST N90606 by Seed et al.

Aside from the N90606 and M78230 sequences explicitly referred to in item 2 of the Office Action, we would like to bring to the attention of the Examining Division to a third prior art sequence, namely Genbank Accession No. Z60980 (Genbank Report and alignment enclosed). This sequence aligns to a region of 109 nucleotides in the coding region of the nucleotide sequence in Figure 1 (SEQ ID NO.:1). The Z60980 sequence was cloned because it contains a CpG island. CpG islands are short stretches of DNA containing a high density of non-methylated CpG dinucleotides, that are known to overlap the 5' end of mRNA transcripts. Neither the Genbank Report nor the associated Cross et al. article (enclosed) discloses a protein comprising at least 30 amino acids of SEQ ID NO.:2 capable of stimulating an immune response. Accordingly, new claim 1 is novel and inventive over the Z60980 sequence.

3. Inventive step (Art. 56 EPC) of previous and new claim 1(f)


The Examining Division took the position that previous claim 1(f) lacked inventive step because the fragments encompassed by said alternative of claim 1 would not solve the technical problem underlying the present invention.

This objection does not apply for new claim 1(f). The fragments have been further defined as being capable of stimulating an immune response as described on page 46, third paragraph, of the description.

4. Request

With the above explanations and the proposed modifications to the claims and the description, Applicant has met the requirements as set forth in the Official Communication.

If, however, the Examining Division does not agree to the above, it is requested that either a further Communication pursuant to Article 96(2) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the telephone.



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosure:
As mentioned above

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polypeptide encoded by a polynucleotide of any one of (a) to (d) wherein said fragment is capable of stimulating an immune response; and
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence.
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response;
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

4. The polynucleotide of any one of claims 1 to 3 which is fused to a heterologous polynucleotide.
5. A vector containing the polynucleotide of any one of claims 1 to 4.
6. The vector of claim 5 in which the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
7. A host cell genetically engineered with the polynucleotide of any one of claims 1 to 4 or the vector of claim 5 or 6.
8. A process for producing a polypeptide capable of stimulating an immune response comprising: culturing the host cell of claim 7 and recovering the polypeptide encoded by said polynucleotide from the culture.
9. A process for producing cells capable of expressing a polypeptide which is capable of stimulating an immune response comprising genetically engineering cells with the vector of claim 5 or 6.
10. A polypeptide having the amino acid sequence encoded by a polynucleotide of any one of claims 1 to 4 or obtainable by the process of claim 8.
11. An antibody specific for the polypeptide of claim 10.
12. A nucleic acid molecule which specifically hybridizes to a polynucleotide of any one of claims 1 to 4.
13. An antagonist/inhibitor of the polypeptide of claim 10, wherein said antagonist/inhibitor is an antibody of claim 11 capable of inhibiting or extinguishing the activity of the polypeptide of claim 10 or a nucleic acid molecule of claim 12 capable of binding and thereby inhibiting the expression of the polynucleotide or the DNA of any one of claims 1 to 4.

14. A pharmaceutical composition comprising the polynucleotide of any one of claims 1 to 4, the polypeptide of claim 10 or a DNA encoding and capable of expressing said polypeptide in vivo or the antagonist/inhibitor of claim 13 and optionally a pharmaceutically acceptable carrier.
15. A diagnostic composition comprising the polynucleotide of any one of claims 1 to 4, the nucleic acid molecule of claim 12 or the antibody of claim 11.
16. Use of the polypeptide of claim 10 or the polynucleotide of any one of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment of neoplasia, for wound-healing, for the treatment of restenosis, for regulating hematopoiesis in endothelial cell development, for stimulating an immune response against parasitic, bacterial or viral infections, or for the treatment and/or prevention of autoimmune diseases.
17. Use of the antagonist/inhibitor of claim 13 for the preparation of a pharmaceutical composition for the treatment of cachexia, cerebral malaria, rheumatoid arthritis, for the prevention of graft-host rejection, for inhibiting bone resorption, for the treatment and/or prevention of osteoporosis, or for the treatment of endotoxic shock.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 10 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 10 in a sample derived from a host.
20. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 10 comprising:
 - (a) contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF

delta polypeptide and a compound under conditions to permit binding to the receptor; and

- (b) determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.

Tumor necrosis factor (TNF- α and TNF- β) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine capable of numerous biological activities including apoptosis of some transformed cell lines, mediation of cell activation and proliferation and also as playing important roles in immune regulation and inflammation.

To date, there are nine known members of the TNF-ligand superfamily, TNF- α , TNF- β (lymphotoxin- α), LT- β , OX40L, FASL, CD30L, CD27L, CD40L and 4-1BBL. The ligands of the TNF ligand superfamily are acidic, TNF-like molecules with approximately 20% sequence homology in the extracellular domains (range, 12%-36%) and exist mainly as membrane-bound forms with the biologically active form being a trimeric/multimeric complex. Soluble forms of the TNF ligand superfamily have only been identified so far for TNF, LT α , and FASL (for a general review, see Gruss, H. and Dower, S.K., *Blood*, 85 (12):3378-3404 (1995)), which is hereby incorporated by reference in its entirety. ESTs have been reported by Seed in EP-A1 330 191 (EST N 90606) and by Adams, *Nature* 355: 632-634 (EST M 78230). In addition, a genomic DNA fragment has been deposited under 260780.

These proteins are involved in regulation of cell proliferation, activation, and differentiation, including control of cell survival or death by apoptosis or cytotoxicity (Armitage, R.J., *Curr. Opin. Immunol.*, 6:407 (1994) and Smith, C.A., *Cell*, 75:959 (1994).

TNF is produced by a number of cell types, including monocytes, fibroblasts, T cells, natural killer (NK) cells and predominately by activated macrophages. TNF- α has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, resistance to parasites, producing an anti-viral response, septic shock, growth regulation, vascular endothelium effects and metabolic effects. TNF- α also triggers endothelial cells to secrete various factors, including PAF-1, IL-1, GM-CSF and IL-6 to promote cell proliferation. In addition, TNF- α up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1.

understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only.

~~Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.~~

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings depict certain embodiments of the invention. They are illustrative only and do not limit the invention otherwise disclosed herein.

Figure 1 shows the nucleotide and deduced amino acid sequence of human TNF delta.

Figure 2 shows the nucleotide and deduced amino acid sequence of human TNF epsilon.

Figure 3 shows the regions of similarity (alignment report) between amino acid sequences of TNF α , TNF β , TNF δ and TNF ϵ polypeptides.

Figure 4 shows structural and functional features of TNF delta deduced by the indicated techniques, as a function of amino acid sequence.

Figure 5 shows structural and functional features of TNF epsilon deduced by the indicated techniques, as a function of amino acid sequence.

The following illustrative explanations are provided to facilitate understanding of certain terms used frequently herein, particularly in the examples. The explanations are provided as a convenience and are not limitative of the invention.

Engineered fibroblasts then may be injected into rats, either alone or after having been grown to confluence on microcarrier beads, such as cytodex 3 beads. The injected fibroblasts produce TNF delta or TNF epsilon product, and the biological actions of the protein are conveyed to the host.

~~It will be clear that the invention may be practiced otherwise than as particularly~~
described in the foregoing description and examples. Numerous modifications and
variations of the present invention are possible in light of the above teachings and,
~~therefore, are within the scope of the appended claims.~~



Blast 2 Sequences results

PubMed

Entrez

BLAST

OMIM

Taxonomy

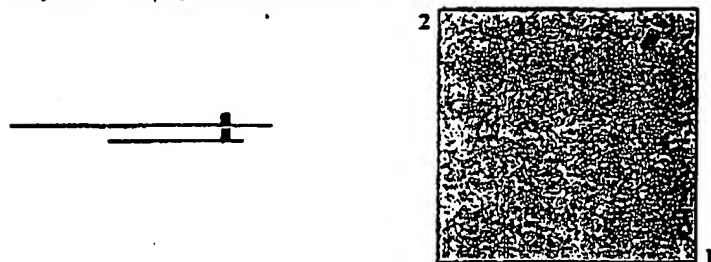
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Gapped

Lambda K H
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S2: 21 (41.1 bits)



Blast 2 Sequences results

PubMed

Entrez

BLAST

OMIM

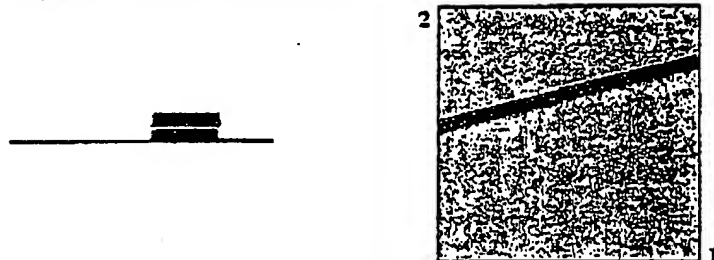
Taxonomy

Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

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NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

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 Strand = Plus / Plus

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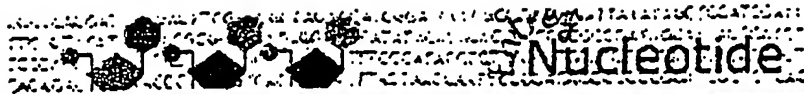
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Gapped
 Lambda K H
 1.33 0.621 1.12

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 effective search space used: 1667565454856
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 A: 0
 X1: 6 (11.5 bits)
 X2: 26 (50.0 bits)
 S1: 12 (23.8 bits)
 S2: 20 (39.1 bits)



PubMed	Nucleotide	Protein	Genome	Structure	PopSet	Taxonomy	OMIM
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Display	Genome	Protein	Genome	Structure	PopSet	Taxonomy	OMIM

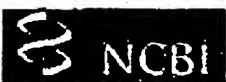
☐ 1: Z60980 H.sapiens CpG isl...[gi:1033358]

Related Sequences, PubMed, Taxonomy

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 forward read cpg40d10.fclla.
 ACCESSION Z60980
 VERSION Z60980.1 GI:1033358
 KEYWORDS CpG island; genomic MseI fragment.
 SOURCE human.
 ORGANISM Homo sapiens
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
 Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
 REFERENCE 1 (bases 1 to 288)
 AUTHORS MacDonald, M., Huckle, E., Wilkinson, P. and Micklem, G.
 TITLE Direct Submission
 JOURNAL Submitted (16-OCT-1995) The Sanger Centre, Hinxton, Cambridgeshire,
 CB10 1RQ, England. E-mail contact: humquery@sanger.ac.uk
 REFERENCE 2 (bases 1 to 288)
 AUTHORS Cross, S.H., Charlton, J.A., Nan, X. and Bird, A.P.
 TITLE Purification of CpG islands using a methylated DNA binding column
 JOURNAL Nat. Genet. 6 (3), 236-244 (1994)
 MEDLINE 94282070
 COMMENT Vector: pGEM-52f(-)
 Clones are available from the UK MRC Human Genome Mapping Project
 Resource Centre, Hinxton, Cambridgeshire CB10 1RQ, UK. See URL:
 http://www.hgmp.mrc.ac.uk/ for details
 or contact: biohelp@hgmp.mrc.ac.uk.
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 121 accgacacac tctcaccctc agatgactcc gatgtgacag aggtgatgtg gcaaccagct
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 241 gttcatctgc ttgtatngcc agggtaacct cagccacact ctgagctt

//

Restrictions on Use | Write to the HelpDesk
 NCBI | NLM | NIH



Blast 2 Sequences results

PubMed

Entrez

BLAST

OMIM

Taxonomy

Structure

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

Match: Mismatch: gap open: gap extension:
 x_dropoff: expect: wordsize: Filter: ☐

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Sequence icl|Figure1EP96910483.5 Length 1717 (1..1717)



NOTE: The statistics (bitscore and expect value) is calculated based on the size of nr database

Score = 216 bits (112), Expect = 1e-53
 Identities = 120/122 (98%), Gaps = 1/122 (0%)
 Strand = Plus / Plus

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Query: 202 caggcccaaggatatagtgtccgaatccaggatgctggagcttctctgtatngcca 261

Sbjct: 726 caggcccaaggatatagtgtccgaatccaggatgctggagcttctctgtatngcca 784

Query: 262 gg 263

Sbjct: 785 gg 786

CPU time: 0.04 user secs. 0.05 sys. secs. 0.09 total secs.

Gapped
 Lambda K H
 1.33 0.621 1.12

Gapped
 Lambda K H
 1.33 0.621 1.12

Matrix: blastn matrix:1 -2
 Gap Penalties: Existence: 5, Extension: 2

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Number of sequences better than 10.0: 1
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X2: 26 (50.0 bits)
S1: 12 (23.8 bits)
S2: 19 (37.2 bits)

Purification of CpG islands using a methylated DNA binding column

Sally H. Cross, Jillian A. Charlton, Xinsheng Nan & Adrian P. Bird

CpG islands are short stretches of DNA containing a high density of non-methylated CpG dinucleotides, predominantly associated with coding regions. We have constructed an affinity matrix that contains the methyl-CpG binding domain from the rat chromosomal protein MeCP2, attached to a solid support. A column containing the matrix fractionates DNA according to its degree of CpG methylation, strongly retaining those sequences that are highly methylated. Using this column, we have developed a procedure for bulk isolation of CpG islands from human genomic DNA. As CpG islands overlap with approximately 60% of human genes, the resulting CpG island library can be used to isolate full-length cDNAs and to place genes on genomic maps.

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The mammalian genome can be conveniently divided into two fractions with respect to DNA methylation^{1,2}. In the major fraction (about 98% of the total) the dinucleotide CpG occurs on average every 50 to 100 basepairs (bp) and is heavily methylated. In the minor fraction (about 2%), CpG occurs approximately every 10 bp and is non-methylated. Although the minor fraction is only a small proportion of the total, it is of great interest, as it is distributed throughout the genome in 45,000 short regions of about 1 kilobase (kb), known as CpG islands. These colocalize with the 5' end of genes³⁻⁵. In humans, about 60% of genes are associated with CpG islands, including all housekeeping genes so far analysed and about 40% of tissue-restricted genes^{6,7}. In most cases, the island contains the promoter and one or more exons of its associated gene.

A representative library of CpG islands would be useful for genome analysis in a number of respects. It would provide an unbiased collection of DNA segments corresponding to the promoters of about 60% of human genes. Moreover, as islands invariably overlap the 5' ends of transcripts, appropriate cDNA clones could be selected, thereby linking cDNAs and genomic DNA at a discrete site. Screening in this way would preferentially select full-length cDNA clones, and could therefore provide complete sequences of both the transcript and its promoter. Furthermore CpG islands contain most of the sites at which rare-cutting restriction enzymes cleave genomic DNA⁸ and thus are landmarks for physical mapping of the genome.

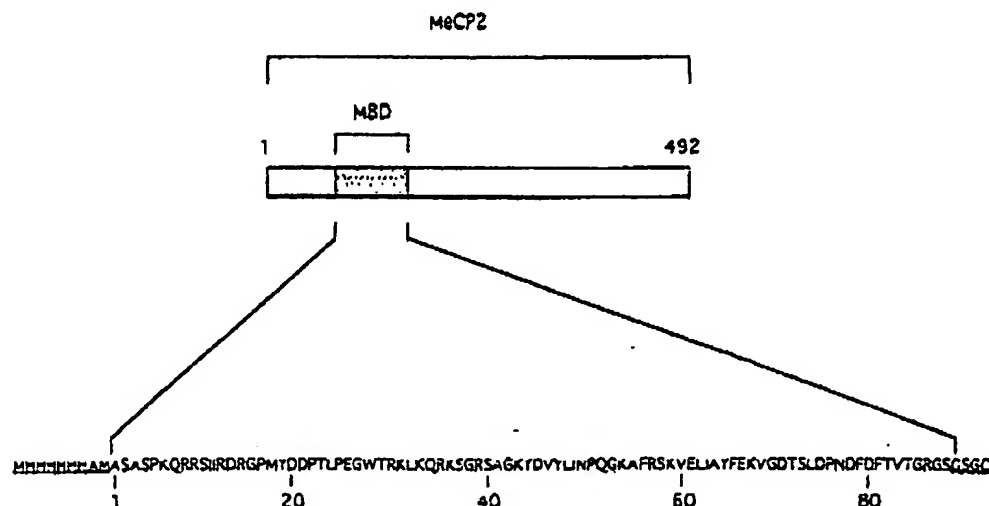
Methods have been developed to isolate the rare-cutting restriction enzyme sites within CpG islands but these are destructive and cannot be adapted to mass isolation of

intact islands. To circumvent this problem, we have developed a non-destructive procedure. The method makes use of the rat chromosomal protein, MeCP2, which binds DNA that is methylated at CpG⁹. The native protein also has a non-specific affinity for DNA¹⁰, but this can be eliminated by gene manipulation, leaving only the methyl-CpG binding domain¹¹ (MBD). The MBD is an 85 amino acid polypeptide that binds to a single symmetrically methylated CpG pair. Here we show that the MBD column can be used to separate DNA solely on the basis of its degree of methylation. This has allowed us to purify and clone the CpG island fraction from human DNA.

Fractionation of DNA using an MBD column
DNA encoding the MBD from MeCP2 was amplified by the polymerase chain reaction (PCR) and cloned into the bacterial expression vector, pET6H (gift from C. H. Hu). The vector contains a sequence coding for a tract of six histidines upstream of the cloning site so that the expressed recombinant protein (rMBD) carries a histidine tag at its N-terminal end, fused with the MBD (Fig. 1). The fusion protein was attached to a nickel-agarose matrix via the poly-histidine tag¹².

To assess the column, we tested whether differentially methylated forms of the same DNA fragment could be resolved. Plasmid pCG11 (ref. 14) was linearized and modified to give three forms: unmethylated, methylated at 37 CpGs within *HhaI* sites, or methylated at all 200 CpGs (Fig. 2a). The fragments were end-labelled and a mixture of each form was applied to the column and eluted with a salt gradient. Three peaks of radioactivity, I, II and III, eluted at about 0.4 M, 0.5 M and 0.6 M salt respectively (Fig. 2b). DNA from each peak was cut with

Fig. 1 Sequence of the methyl-CpG binding domain. Schematic diagram of MeCP-2 showing the position of the methyl-CpG binding domain (MBD) as a stippled box. The sequence of the recombinant hMBD protein is shown below. Amino acids derived from the plasmid vector sequences are underlined. hMBD has a molecular weight of 11.4 kD.



the methylation-sensitive restriction enzymes *HpaII* or *CfoI* (an isoschizomer of *HhaI*) and fractionated on a gel (Fig. 2c). Peak I contained DNA that was cut by both *HpaII* and *CfoI*, indicating that it is completely unmethylated. Peak II DNA was cut by *HpaII* but not *CfoI*, indicating that it is partially methylated. Peak III DNA was cut by neither enzyme, indicating that it is completely methylated. Thus the differentially methylated versions of pCG11 were separated by the MBD column.

Binding affinity on the column depended on methyl-CpG density because a DNA molecule containing 1 methylated CpG per 76 bp (*M. HhaI*-methylated pCG11) was bound less tenaciously than one containing 1 methylated CpG per 14 bp (*M. SstI*-methylated pCG11). This was unexpected, as each MBD molecule apparently interacts with a single methyl-CpG pair¹². The tight binding of multiple methylated DNA molecules presumably involves interaction of one DNA molecule with several different MBD moieties. The results also show that the MBD has a weak non-specific DNA-binding activity, as non-methylated DNA is retained on the column at low salt concentrations. This is not due to binding of DNA to the column matrix, which has no affinity for DNA (data not shown), but could be due to the basic nature of the protein (predicted pI 9.75). Non-specific DNA binding is not affected by DNA length, as non-methylated fragments ranging from 0.5 to 23 kb all eluted at close to 0.4 M salt (data not shown).

Separation CpG of islands

The above experiments used artificially methylated constructs. We next tested the behaviour of human genomic DNA by asking if methylated CpG islands from the inactive X chromosome could be resolved from their non-methylated counterparts on the active X chromosome. Female and male human DNA was digested with *MseI*, which cuts bulk DNA into small fragments (100-200 bp average), but leaves CpG islands relatively intact (see below). When *MseI*-digested male or female

salt, and only a small proportion (about 1-2%) bound tightly. Eluted fractions were run on an agarose gel, blotted and hybridized to a CpG island probe from the X-linked monoamine oxidase A gene¹³ (Fig. 3). This CpG island eluted in low salt fractions for both male and female DNA samples. However with female DNA the CpG island also eluted in high salt fractions. It seemed likely that these fractions contained the methylated CpG island from the inactive X chromosome. This was verified by digesting the fractions with the methylation-sensitive restriction enzyme, *SstII*. The monoamine oxidase A CpG island fragments eluting at low salt were cut by this enzyme, but the fragments eluting at high salt were resistant to cleavage (data not shown). The same result was obtained with CpG island probes for the phosphoglycerate kinase gene¹⁴ and a CpG island that lies close to the factor VIII gene¹⁵. Both of these sequences are X-linked and methylated on the inactive X chromosome. They are fractionated as one peak in male DNA and two peaks in female DNA (data not shown). As a control, an autosomal CpG island probe from the α -globin gene was hybridized to the same male and female fractions. A single low salt peak was obtained in each case (data not shown), as expected for a CpG island that is non-methylated on both autosomal chromosomes¹⁶.

Preparation of a CpG island library

Having demonstrated that it is possible to separate methylated and non-methylated CpG islands in the presence of a vast excess of genomic DNA, we decided to use the column to purify CpG islands *en masse*. The strategy can be divided into four steps (Fig. 4a). In the first step, total genomic DNA is fragmented with *MseI*. This enzyme was chosen because its recognition site (TTAA) is found relatively rarely within CpG islands (once per 1,000 bp) but frequently in bulk DNA (once per 140 bp). Therefore *MseI* is expected to give predominantly intact CpG islands, plus small fragments from bulk DNA. To illustrate this, Fig. 4b shows the restriction map of a 58 kb DNA sequence derived from human chromosome 4p16.3.

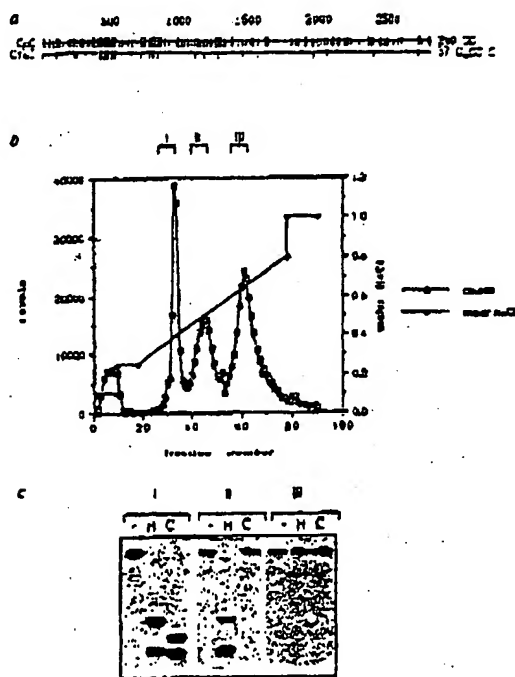


Fig. 2 Fractionation of differentially methylated pCG11 molecules. a, The position and number of methylated CpGs in either the completely methylated (top line) or partially methylated (bottom line) form of pCG11 are shown. Plasmid pCG11 was methylated to completion using either M SssI or M HpaII methyltransferase. b, A mixture of fully methylated, partially methylated and non-methylated linear pCG11 was applied to a 2 ml MBD column and eluted with a gradient of NaCl as shown. 1 ml fractions were collected. The three eluted peaks I, II and III are bracketed. c, Pooled DNA fractions from each of the peaks I, II and III were digested with the methylation-sensitive restriction enzymes shown and fractionated on a 2% agarose gel. The gel was dried and exposed to X-ray film. -, undigested; C, CfoI; H, HpaII.

The pattern of frequent *MseI* sites is interrupted by three gaps: these correspond to three CpG islands as shown by clustered sites for *NarI*, *EagI* and *BstXI*.

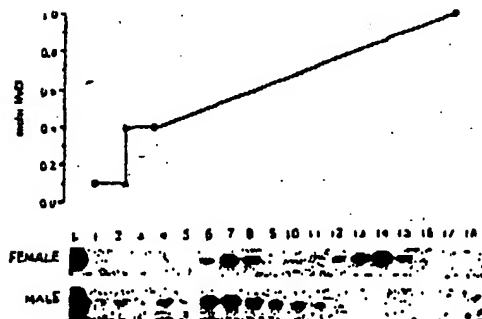
The second step in the protocol involves removal of *MseI* fragments that contain clusters of methylated CpGs, by selecting only fragments that bind weakly to the column. This "stripping" procedure is required to remove highly methylated *MseI* fragments that would contaminate the final purified CpG island fraction (see below). The third step makes use of the bacterial methyltransferase, *M. SssI*, to methylate all non-methylated CpGs in the fractions that have been stripped. On most fragments, nearly all CpGs are methylated already and therefore their affinity for the MBD column is not changed. CpG island fragments, on the other hand, are converted from weak-binding to strong-binding molecules. By now selecting the fragments that elute at high salt, step four should yield a fraction that is highly enriched for CpG islands.

Fig. 3 Separation of methylated and non-methylated CpG islands from the active and inactive X chromosomes. a, 50 µg of *MseI*-digested human male or female DNA derived from blood was loaded onto a 1 ml MBD column. The column was washed with a salt gradient as shown. Fractions of 4 ml were collected. b, DNA from each fraction was separated on a 1.3% agarose gel and transferred to Hybond-N+. The results of hybridizing the filter with a probe for the monoamine oxidase A (MAOA) CpG island is shown for female DNA (top panel) and male DNA (bottom panel). L, Load DNA. Numbers correspond to fractions collected off the column.

The strategy was tested on human male genomic DNA. The fate of *MseI* fragments corresponding in three different classes of sequence was assayed using the PCR (Fig. 4c). The test sequences comprised a CpG island fragment of the monoamine oxidase A gene (MAOA), a fragment containing a cluster of methylated CpGs that is located at the 3' end of the human apolipoprotein A-IV gene¹⁹ (APOA4), and two fragments representative of bulk DNA that contain only a few CpGs (ALDOB and IFN γ). In practice, three passes of the *MseI*-cut DNA were necessary to strip all of the heavily methylated APOA4 fragment. In each stripping step, the CpG island (MAOA) and bulk genome fragments (ALDOB and IFN γ) co-eluted at low-salt. Following *de novo* methylation the DNA was passed over the MBD column again. During these "binding" steps the ALDOB and IFN γ fragments were in the unbound fraction as before, but the MAOA CpG island fragment now switched from the unbound to the bound fraction. After two cycles of binding, the only fragment detectable by the PCR in the bound fraction was from the MAOA CpG island. The other three monitored fragments had been lost. DNA from the final bound fraction was cloned into the *NdeI* site of pGEM-52R(-).

Proportion of CpG island-like clones

In order to estimate the proportion of clones containing sequences derived from CpG islands, we amplified inserts from the clones using primers which flanked the cloning site, and tested them for the presence of *BstXI* sites. *BstXI* recognizes the sequence CGCG which occurs frequently within CpG islands (once per 90 bp), but is rare in bulk



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DNA (one site per 5,000–10,000 bp; see Fig. 4b) Inserts containing *Bst*UI sites were considered likely to be derived from CpG islands. Altogether 113 clones were analysed, of which 87 (77%) contained inserts with *Bst*UI sites (see Fig. 5). The average size of the *Bst*UI-containing inserts was 760 bp. This is somewhat smaller than the average size of a CpG island (1 kb) and probably reflects the proportion of CpG islands that contain an *Mse*I site. A survey of 80 CpG islands from the EMBL sequence database showed that 38% do contain an *Mse*I site (S.H.C., unpublished observations).

As a more thorough test of their identity, the DNA sequence of eighteen clones was determined. The base composition (% G+C) of each insert and the ratio of observed and expected frequencies (O/E) of the dinucleotide CpG are plotted in Fig. 6. CpG islands typically have a base composition in excess of 50% G+C, and an observed frequency of CpG that is close to the expected frequency (O/E typically equals about 0.85).

They are therefore expected to cluster in the upper right quadrant of Fig. 6. Bulk chromosomal DNA, on the other hand, has an average base composition of 40% G+C and about 20–25% of the expected frequency of CpG (O/E = 0.22; "Bu" on Fig. 6). It will therefore fall into the lower left quadrant of the graph. For example, clone 1, which had no *Bst*UI sites, falls close to the expected position of bulk DNA in Fig. 6. All the other inserts lie close to positions expected for CpG islands.

In addition to their unusual primary sequence, CpG islands are also characterized by lack of methylation in an

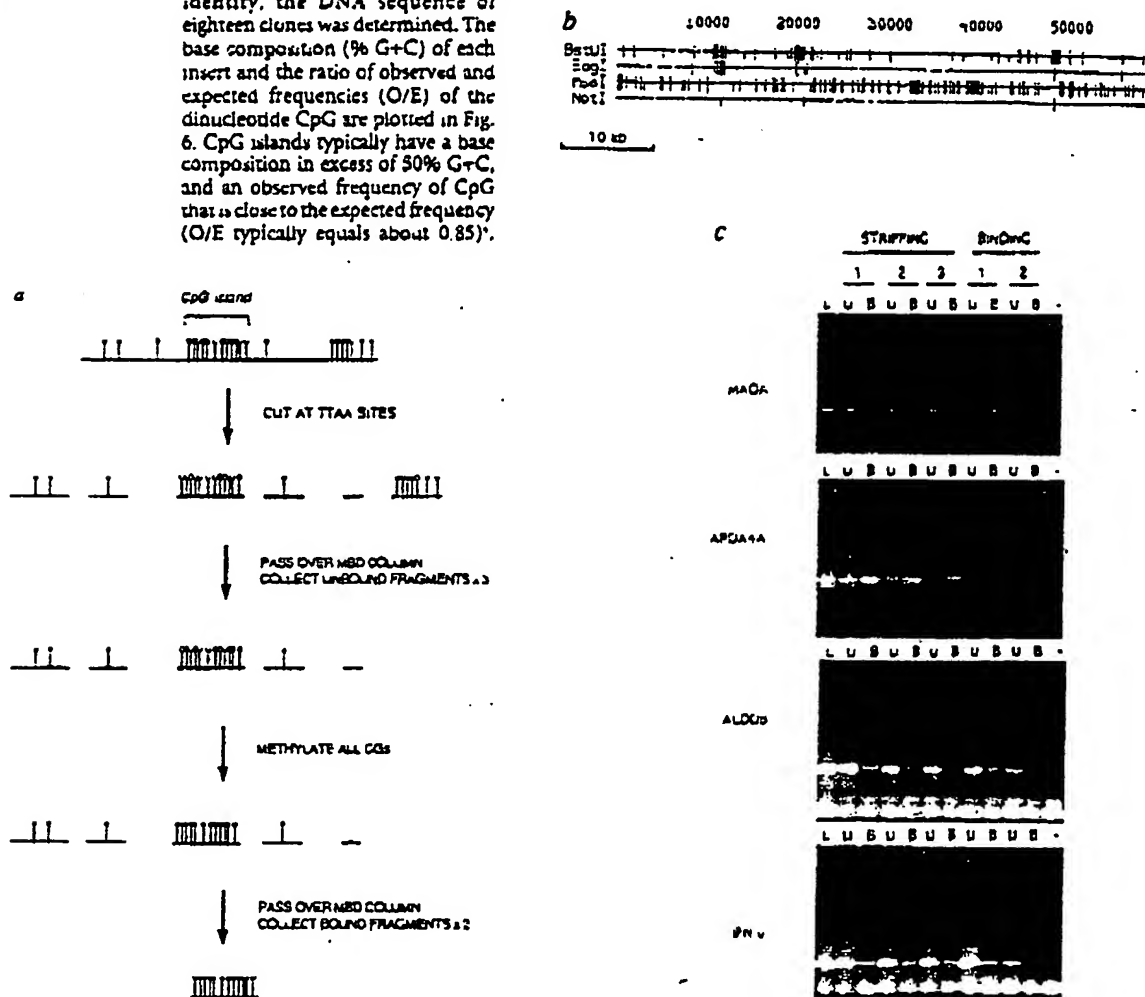


Fig. 4 Construction of a CpG island library. **a**, Flow diagram illustrating the strategy for the purification of *Mse*I fragments containing CpG islands. The position of CpGs is indicated by vertical lines. Open and solid circles denote unmethylated and methylated CpGs, respectively. **b**, Position of sites for the restriction enzymes *Bst*UI, *Eag*I, *Mse*I and *Not*I in a 58 kb region of human chromosome 4p18.3 (ref. 39). **c**, The rate of *Mse*I fragments corresponding to three classes of sequence during the stripping and binding steps of CpG island purification. The three classes were (i), CpG island (MAOA, monoamine oxidase A CpG island); (ii), a sequence with a cluster of methylated CpGs (APOA4, 3' exon of apoA-IV gene); and (iii), CpG-deficient sequences (ALDOB = aldolase B gene fragment, IFN γ , interferon γ gene fragment). PCR amplification was carried out on samples from human male *Mse*I-digested DNA (L), fractions eluting up to 0.4 M NaCl (U), fractions eluting at 0.6 M NaCl (B) and control blank reactions (-). Reactions were carried out for the three "stripping" steps and the two "binding" steps (see Methodology).

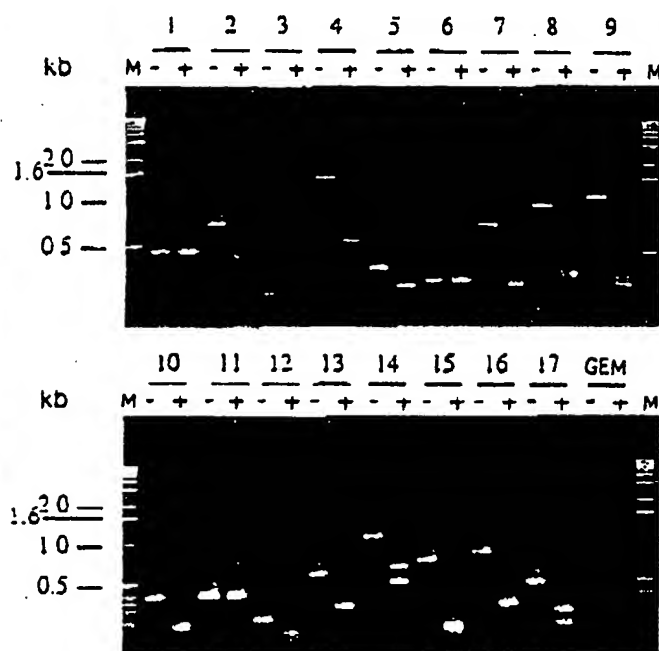


Fig 5 BstUI Analysis of clones. Inserts from random clones were amplified by the PCR and digested with BstUI. The digests were separated on 2% agarose gels and stained with ethidium bromide. For each clone both undigested (-) and digested (+) insert DNA is shown. GEM, vector alone; M, marker. Sizes are in kb.

otherwise heavily methylated genome¹. We examined whether clones in the library were derived from unmethylated parts of the genome by using 20 random inserts as probes against blots of human male DNA that had been digested with *Hpa*II, *Msp*I or *Bst*UI (Table 1). Thirteen of the clones detected single copy fragments that were cut identically by *Hpa*II and *Msp*I. Twelve of these were also cleaved by *Bst*UI; the thirteenth, clone 7, did not contain any *Bst*UI sites. Three further clones detected up to five fragments, all of which contained unmethylated *Bst*UI and *Hpa*II sites. Two clones also detected a small number of fragments (in addition to a background smear of hybridization), but in these cases not all of the fragments were unmethylated. For one of these, clone A8, a fragment of the same size as the insert contained unmethylated *Bst*UI and *Hpa*II sites. Thus in 17 out of 20 cases, the cloned inserts detected genomic fragments that contained non-methylated sites for *Hpa*II and *Bst*UI (when present). Of the remaining three probes, one (clone A10) was obscured by background hybridisation, one (clone 8) was highly repeated, and one (clone 1) had no testable sites and had sequence properties resembling bulk genomic DNA (see above and Fig 6). The sequence and Southern data taken together suggest two things. Firstly, about 80% of clones in this library are derived from non-methylated CpG-rich sequences which have the characteristics of CpG islands; and secondly, most clones in the library are derived from sequences which are single-copy in genomic DNA.

rRNA genes and repetitive elements in the library
There are about 100 repeat units of the ribosomal RNA genes (rDNA) in the haploid human genome, all of which are non-methylated and CpG rich²⁰. We expected that rDNA would behave in the same way as CpG islands on the column, and would therefore be represented in the library. To check this, 375 clones were gridded onto membranes and hybridized to the complete rDNA repeat unit. Table 2 shows that 47 clones hybridized, representing 12.5% of the library. The expected proportion of rDNA clones can be simply estimated by taking the total length of CpG island DNA per haploid genome as 4.5×10^7 bp (45,000 islands each of 1 kb²¹) and the total length of rDNA as 4.5×10^6 bp (100 repeat units each of 45 kb²¹). It is apparent that 10% of clones in the CpG island library are expected to be rDNA, which is close to the observed figure of 12.5%.

The same set of gridded clones was probed with total human DNA, an *Alu* repeat and a L1 repeat, in order to determine the frequency of highly repetitive elements in the library (Table 2). In all, 10% of clones hybridized to these probes, and significantly, the majority of these (28 out of 36) also hybridized to rDNA. As the rDNA repeat unit is known to contain dispersed repeat elements, the repeat-positive clones may be *bona fide* rDNA sequences. Alternatively, they may be repeat elements from elsewhere in the genome that anneal with related elements in the rDNA unit. We favour the former explanation, as the number of rDNA-positive clones in the library is close to the expected level, and agrees with previous data in which only the rDNA transcription unit was used as a probe⁴. If indeed repetitive elements in the library are predominantly due to rDNA, then the genuine CpG island clones, which account for about 80% of this library, are remarkably free of dispersed repeats. This agrees with the results of Southern analysis (Table 1) which showed that 16 out of 19 CpG island-like clones were single-copy or close to it.

Transcripts associated with CpG island clones.
A significant reason for preparing CpG island libraries is to obtain full length cDNAs of the associated gene.

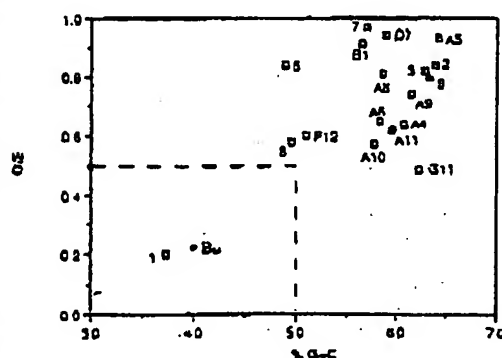


Fig 6 Scatter plot showing sequence characteristics of the cloned DNA fragments. The % G-C content (% G-C) and the observed/expected number of CGs (O/E) are plotted. Open squares show the position for each clone. A diamond marks the typical position of bulk (Bu) DNA. The lower left-hand quadrant in which bulk DNA would be expected to fall is boxed by a dashed line.

Genomic sequences in the database show clearly that CpG islands overlap the 5' ends of transcripts (Fig. 7). We surveyed the ability of clones from the library to detect mRNAs by using them as probes on northern blots. In order to distinguish genuine transcripts from spurious cross-hybridisation to 28S and 18S rRNA, inserts were hybridized to both total RNA and poly-A⁺ RNA from the human male lymphoblastoid cell-line, PES². Messenger RNA signal should anneal more strongly in the poly-A⁺ lane than in the total RNA lane, whereas the opposite is expected of a probe that detects rRNA. Of 18 clones (discounting clone 1 which was not island-like, and clone 8 which contained an Alu repeat), five inserts detected discrete transcripts in poly A⁺ RNA from PES and none hybridized with rRNA (Table 1).

We also screened the DNA sequence database with 18 insert sequences, and obtained five strong matches (Table

1). Clone 7, which detected a transcript by hybridization, had identity with the 5' end of the human 39 kD nuclear-encoded NADH-ubiquinone oxidoreductase sub-unit²⁴ (Fig. 8a,b). The cDNA sequence in the database is incomplete and lacks an initiator ATG codon. The island clone extends further 5' and contains an in-frame ATG. Comparison with the bovine cDNA sequence²⁵ shows that this is highly likely to be the correct start codon. At its 3' end the homology ends at a GT dinucleotide which probably represents the 5' boundary of an intron. Clone A5 has identity with the 5' end of an expressed sequence tag (EST) from a human infant brain cDNA library²⁶ (Fig. 8c,d). Once again, the island clone provides more 5' sequence than is present in the database sequence, and may include the transcription start and promoter sequences of this unknown gene. For the third match, clone A10 has identity with an *Msd* fragment from the β -3-adrenergic receptor promoter²⁷ (EMBL X72861, bases 2162-2328). The other two matches were to human mitochondrial DNA (clone F12) and to Alu repeats (clone 8). As the library was constructed with DNA from whole blood, the presence of mitochondrial sequences, which contain no detectable m⁵C²⁸, is not unexpected. Out of 23 clones that were analysed in detail, six detected mRNAs in either lymphoblastoid cells or in the sequence database.

Discussion

We have developed a novel affinity column that fractionates DNA according to its level of m⁵CpG methylation. The column can be used analytically or preparatively and has a number of potential applications. We have used it to prepare a library of CpG islands from human male genomic DNA. Initial characterization of the library indicates that about 80% of clones derive from CpG island-like sequences, 10% are rDNA and the rest are bulk genomic or mitochondrial DNA contaminants. The proportion of island clones is therefore high, and may be further improved in future libraries of this kind (henceforth referred to as "CGI libraries"). Mean insert size is 760 bp, which is somewhat smaller than the genomic average for CpG islands (1 kb²⁹). This discrepancy is most probably due to the presence of a mixture of intact CpG islands and islands that have been cut on average once by *MspI*. Of 80

Table 1 Analysis of 23 clones

Clone name	Insert size (bp)	BstRI sites ^a	Southern ^b	Northern ^c	Sequence database searches ^d
1	418	+	sc	-	-
2	664	+	sc	+	-
3	113	+	sc	+	-
4	1813	+	sc	-	nd
6	311	+	sc	-	-
7	228	+	sc	+	39 kD NADH-ubiquinone oxidoreductase sub-unit human
8	534	+	nr	-	Alu repeat
9	693	+	sc	-	-
10	1043	+	sc	-	nd
12	1213	+	sc	-	nd
A4	834	+	sc	-	-
A5	893	+	nr	-	EST06392 human infant brain cDNA
A6	731	+	sc	-	-
A7	670	+	sc	-	nd
A8	431	+	nr	-	-
A9	571	+	nr	+	-
A10	168	+	nr	-	β -3-adrenergic receptor
A11	472	+	sc	-	-
A12	460	+	nr	-	nd
B1	862	+	sc	-	-
D7	377	+	nd	nd	-
F12	472	+	nd	nd	mitochondrial
G11	543	+	nd	nd	-

^aPresence (+) or absence (-) of *BstRI* sites in the insert.

^bThe inserts were hybridized to Southern blots of human male DNA cleaved with *MspI* and subsequently with no further treatment, *HpaII*, *MspI* or *BstRI*. The DNA was fractionated on a 1% agarose gel and transferred to Hybond N+ before hybridization. sc, single copy (one fragment detected), nr, highly repeated (a strong smear of hybridization detected), ir, low repeat (up to five fragments detected), mr, middle repeat (several fragments and a faint background smear of hybridization detected), nd, not done.

^cThe inserts were hybridized to northern blots of total and poly A⁺ RNA from the human lymphoblastoid cell line PES². Transcript detected, +, no transcript detected: nd, not done.

^dThe Genbank and EMBL databases were searched -. No strong database match: nd, not done.

^eClone 1 did not contain any *HpaII* or *HhaI* sites.

^fClone 7 did contain *HpaII* and *HhaI* sites.

^gClone 8 has 84% identity with the Alu consensus sequence³⁰ and belongs to the class IV Alu sub-family.

Table 2 Hybridization analysis of 375 clones

Number of clones	Probe			Total ^a
	Alu ^b	L1 ^c	Ribosomal ^d	
323	-	-	+	-
4	-	+	-	-
19	-	-	+	-
4	-	-	+	+
12	-	+	+	+
16	+	-	+	+

^a"Blurb", a human Alu repeat clone was used as the probe.

^bSequence from the GC-rich 5' end of the L1 repeat (see Methodology for preparation conditions) or a probe consisting of the 1.2 kb, 1.5 kb and 1.8 kb *KpnI* fragments from the L1 repeat³¹ were used as probes. The 5' end of the L1 repeat detected only one clone.

^cSequences from the entire ribosomal repeat were used as the probe (R. Ando and E. M. Southern, unpublished observation).

^dTotal human male DNA was used as a probe.

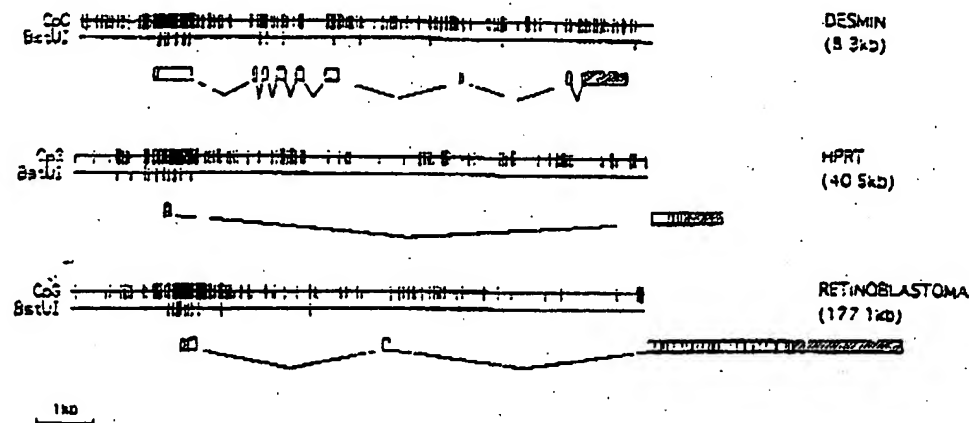


Fig. 7 Diagram showing the structure of three human CpG island genes of different sizes. Vertical lines show the positions of CpGs and Bstul sites in the first 10 kb of the desmin (EMBL ns00501), hprt (EMBL ns0078a) and retinoblastoma (EMBL L11910) genes. The locations of the exons are shown by boxes. Open and hatched portions denote translated and untranslated regions respectively. Any exons not present in the first 10 kb of genomic DNA are shown fused together to the right. The total genomic length of each gene in kb is given in brackets.

CpG island sequences that have been examined, 38% contain an *MseI* site. Assuming 45,000 CpG islands/genome and taking into account those that contain *MseI* sites, a library should contain approximately 60,000 independent clones in order to be representative. On screening 350,000 clones with an X-linked CpG island probe, three positive clones were detected (data not shown). This is the expected number of positives for an X-linked CpG island (six positives would be expected for an autosomal CpG island). Eight randomly chosen CpG islands were also found to be present in the CpG island fraction by Southern blotting and PCR analysis (data not shown). These results indicate that the library is representative.

The level of repeated sequences among CGI clones is much lower than would be expected for total genomic DNA. This agrees with previous results of DNA

reassociation experiments, which indicated that most CpG island sequences are represented 1–5 times in the mouse genome. Southern blot analysis showed that 17 out of 20 CGI clones hybridized to five or fewer fragments in *MseI*-cut genomic DNA, and 14 of these detected a single band. The low frequency of repeats is of biological interest, and may have relevance to the ability of CpG islands to maintain a methylation-free state.

A survey of the EMBL database has shown that about 57% of human genes have CpG islands. Of these, about half are "housekeeping genes" which are expressed in most cell types, and half are "tissue-restricted genes" which are expressed in a subset of cell types. It is evident from published sequences that CpG islands nearly always overlap with the 5' exons of their associated genes. Thus CGI clones should detect mature mRNAs from most human genes by hybridisation. In our analysis 5 out of 20 CGI inserts detected transcripts in lymphoblastoid cell poly-A⁺ RNA. One of these turned out to overlap with the 5' end of the gene for the 39 kD nuclear-encoded NADH-ubiquinone oxidoreductase sub-unit and one further insert that was negative in the northern analysis had identity with the 5' end of a cDNA of unknown function (Table 1). Thus using these limited assays, six transcripts were selected by CGI clones. Evidently the proportion of CGI-positive

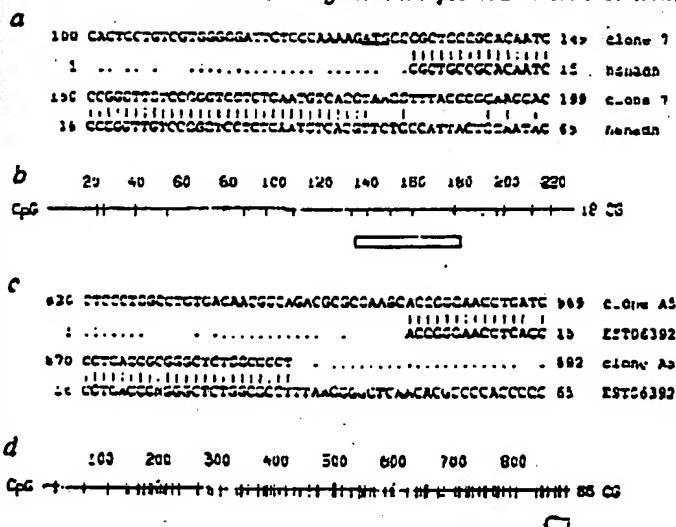


Fig. 8 Database matches for clone 7 and clone A5. a, The sequence match between clone 7 and the 5' end of the 39 kD NADH nuclear-encoded ubiquinone oxidoreductase sub-unit gene (hsnadn, EMBL L04490) is shown. Vertical lines indicate exact matches. The presumed ATG is underlined. b, Diagram of clone 7 showing the positions of CpGs. The box shows the location in clone 7 of the match. The 3' end of the match probably corresponds to an exon/intron boundary. c, The sequence match between clone A5 and EST06392 (EMBL T08501) is shown. Vertical lines indicate exact matches and double dots indicate possible matches. d, Diagram of clone A5 showing the positions of CpGs. The box shows the location in clone A5 of the match.

transcripts would be increased by probing RNA from several human cell types, or from tissues that express a higher proportion of genes, such as brain or testes.

Gene identification via CGI libraries. CGI libraries have potential uses in isolating, mapping and sequencing genes. Because of their small size, sequencing the approximately 45,000 CpG islands in the human genome is a realistic goal. It is also possible that libraries corresponding to fractions of the human genome, for example single chromosomes or YACs, can be made using the column. Whatever the origin, it is expected that a large fraction of the clones will detect mRNAs on northern blots, and can therefore be used to isolate cDNAs. Because CpG islands are usually located at the 5' ends of genes, most cDNAs selected in this way will be complete, or nearly so (see Fig. 7). CpG island fragments have been successfully used to isolate full-length cDNAs^{28,29}, showing the feasibility of this strategy. If the resulting cDNAs were to be sequenced, this would be a once-and-for-all (not a tagging) exercise. The prerequisite, of course, is that the cDNA library should have a high proportion of full-length cDNAs.

The CGI approach to gene identification has significant advantages compared to the more direct cDNA sequencing approach, but it also has some drawbacks. The main drawbacks are that only 60% of human genes have CpG islands (therefore the remaining 40% cannot be detected this way), and that a time-consuming screening step is needed to select cDNAs. A major advantage of the CGI approach is that each CpG island will have the same representation in the library irrespective of the pattern or level of expression of the associated transcript. Other advantages are that the selected cDNAs are likely to be complete (see above), and that each will have a discrete address in the genome, unlike cDNAs which may extend over tens or hundreds of kilobase pairs (see Fig. 7). In most cases the address will include the gene's promoter, for CGI libraries also represent libraries of promoter sequences. Finally as CGI probes are generally single-copy sequences, they make good probes for mapping genes.

Methodology

Preparation of MBD column. The methyl-CpG binding domain of MeCP2¹⁴ was amplified by the PCR using oligonucleotides which introduced an *Nde*I site at the 5' end of the DNA fragment and a *Bam*HI site at the 3' end. The reaction contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl₂, 0.01% gelatin, 0.1% Triton X-100, 200 µM of each dNTP, 1 µM of each primer, 100 ng of a cDNA clone of MeCP2 and 2 U of *Taq* polymerase in a 100 µl reaction mix. The primers used were 5'-GGCCCATGGCTCTCTCTTCTCCAAA-3' and 5'-CCGGCATCCCTCCCTCCCTCCCTTACAGT-3'. 25 cycles of amplification of 1 min at 94 °C followed by 1 min at 50 °C and 3 min at 72 °C were performed. After digestion with *Bam*HI and *Nde*I and gel-purification this fragment was cloned into the *Nde*I and *Bam*HI sites of pET6H, a modified version of pET11D (Novagen) (C.H. Hw, unpublished), to give pET6HMBD. The recombinant protein (HMBD) was expressed in the *E. coli* strain BL21 (DE3) pLysS and was produced essentially as described⁴. Bacterial extract was made by repeated sonication in 5 M urea, 50 mM NaCl, 20 mM HEPES (pH 7.9), 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.5 mM PMSF and the protease inhibitors pepstatin A, leupeptin, chymostatin and antipain each at 5 µg ml⁻¹. After sonication the extract was spun at 15,000 rpm for 30 min in a Beckman JA-20 rotor. The supernatant was dialysed into buffer A (50 mM NaCl, 50 mM phosphate pH 7.0, 10% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol, 0.5 mM PMSF). This was done in several steps. In the first, buffer A included 2.5 M urea and in subsequent steps it included 1.25 M urea, 0.6 M urea, 0.3 M urea and finally no urea. The extract was spun again as above and loaded onto

a 7.5 ml Fractogel EMD SO₃⁻-650(M) column (Merck). The column was washed with buffer A and bound proteins were eluted in buffer A with 1 M NaCl. After dialysis of the eluted fraction into buffer A it was loaded onto a Pharmacia HR 10/10 column containing Fractogel EMD SO₃⁻-650(M) using a Pharmacia FPLC system. The column was washed with buffer A, then buffer A with 0.4 M NaCl followed by a 60 ml linear salt gradient of 0.4–1 M NaCl in buffer A. The HMBD protein eluted between 0.6 and 0.85 M NaCl. The purified protein was coupled to Ni²⁺-NTA-agarose by mixing the protein with the matrix and washing with 8 mM imidazole, 50 mM NaCl, 20 mM HEPES pH 7.9, 10% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol and 0.5 mM PMSF. Approximately 10 mg of HMBD was produced per litre of culture and 18 µg HMBD could be coupled per ml Ni²⁺-NTA-agarose. Heavily methylated DNA fragments elute between 0.6 and 0.85 M NaCl depending on the amount of HMBD on the column.

Separation of DNA on the MBD column. The HMBD protein coupled to Ni²⁺-NTA-agarose was used in Pharmacia HR columns. DNA was loaded in 100 mM NaCl, 20 mM HEPES pH 7.9, 10% glycerol, 0.1% Triton X-100, 10 mM β-mercaptoethanol and 0.5 mM PMSF (buffer B). Bound fractions were eluted using NaCl gradients.

Preparation of the CpG island fraction. Human male *Mel*-digested DNA (100 µg) was loaded onto a column (2.5 mg ml⁻¹ HMBD coupled to Ni²⁺-NTA-agarose in a Pharmacia HR 5/10 column). The column was washed with 8 ml buffer B containing 0.4 M NaCl, then a 40 ml linear salt gradient from 0.4–0.8 M NaCl in buffer B followed by 10 ml buffer B containing 1 M NaCl. 2 ml fractions were collected during the linear gradient. The flow-through, 0.4 M NaCl wash and first two fractions were pooled, ethanol precipitated and reloaded onto the column in buffer B. This step was repeated twice. The DNA remaining after the third pass over the column was methylated using the *M* SssI DNA methyltransferase (New England Biolabs) following the manufacturer's instructions. Efficiency of the methylation reaction was tested by PCR amplification of a fragment from the monomine oxidase a CpG island following digestion with *Hpa*II. When the intensity of the fragment band was undiminished by digestion, it was assumed to be fully methylated. The methylated DNA was loaded onto the column which was then washed as before. Fractions (2 ml) eluting between 0.5 M and 0.75 M NaCl were pooled, dialysed into buffer B, reloaded onto the column, then washed and fractionated as before.

PCR amplification of genomic DNA. The reaction conditions used were as described for the amplification of the MBD fragment with various modifications. For the monomine oxidase a CpG island sequence the reaction buffer was supplemented with 2.5 mM MgCl₂ and 0.5 × 10³ TMAC. The primers used were 5'-CCGGTATCAGATTGAAACAT-3' and 5'-CTCTAAGCATGGCTACACTACA-3'. For the sequence from the 3' end of the apolipoprotein A-IV gene the reaction buffer was supplemented with 10% DMSO. The primers used were 5'-GGAGAAGTGAACACTTACCC-3' and 5'-TTTGAAATTCGTGACGGTAC-3'. The sequences from the aldolase band and interferon α genes¹⁴ were amplified by the PCR as described⁴. For the 5' end of the L1 repeat the primers used were 5'-CTCTGCAGAGCTTACTGCTGTCTT-3' and 5'-CCAAATGCGCCGAATAGGAACAGC-3'. In all cases 30 cycles of amplification (1 min at 94 °C, 2 min at 55 °C and 3 min at 72 °C) were performed.

Cloning and analysis of the CpG island fraction. *Mel*I fragments were cloned into the *Nde*I site of pGEM-32R(-) (Promega) and transformed into the methylation-tolerant *E. coli* strain XL1-BLUE MRF' (Stratagene). To amplify the inserts colonies were picked, and streaked onto a fresh ampicillin plate. The remainder of each colony was mixed with 100 µl water, boiled for 5 min and spun to remove cell debris. 10 µl of this solution was used for PCR. PCR reactions were carried out in buffer III³⁰ supplemented with 10% DMSO in a total volume of 100 µl. 30 cycles of amplification (1 min at 94 °C, 1 min at 55 °C and 3 min at 72 °C) were performed. The primers flanked the *Nde*I site and were 5'-CCGGCCGCTGCAAGCTGACCTTAA-3' and 5'-AACCGCTTGGGAGCTCCTCCCTAA-3'. Clones were sequenced from forward, reverse or custom-made primers (IDF DNA synthesis service) using the dideoxy chain termination method and either a USB DNA sequencing

but an Applied Biosystems 373A DNA sequencer. The sequences have been deposited in the EMBL Data Library. The clones have been assigned the following accession numbers: 1. X76662, 2. X76663, 3. X76664, 7. X76665, 8. X76666, 9. X76667, 11. X76668, 10. X76669, 14. X76670, 15. X76671, 16. X76672, 18. X76673, 19. X76674, 21. X76675, 22. X76676, 23. X76677, 24. X76678, 25. X76679, 26. X76680, 27. X76681.

Southern and northern blot analysis. DNA, total RNA and poly A⁺ RNAs were prepared by standard procedures. DNA fragments were separated on agarose gels and transferred to Hybond-N⁺ (Amersham) as recommended by the manufacturer. Filters were hybridized as described¹ and after hybridization washed at 65 °C with 0.2x SSC. 0.1% SDS RNAs were separated on formaldehyde gels and transferred to Hybond-N⁺. Filters were hybridized in 0.33 M Na₂HPO₄ (pH 7.2), 7% SDS, 30% formamide, 1 mM EDTA (pH 8.0) and 1% BSA at 60 °C. Filters were washed at 60 °C with 0.4x SSC, 0.1% SDS. All probes were labelled by the random priming method². After hybridization and washing, filters were exposed to X-ray film for autoradiography or were analyzed using a phosphorimager (Molecular Dynamics).

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Computer analysis. All sequence analysis was carried out using the University of Wisconsin sequence analysis software package³ and the BLAST program⁴.

Library distribution. The library, named human CGII (human CpG island library 1), will be distributed by the HGMZ Resource Centre, Clinical Research Centre, Watford Road, Harrow HA1 3UJ, U.K.

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Application No. 96 910 483.5-2110	Ref. C 2276 EP	Date 23.08.2002
Applicant HUMAN GENOME SCIENCES, INC.		

Communication pursuant to Article 96(2) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(1) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 78(2) and 83(2) and (4) EPC.

One set of amendments to the description, claims and drawings is to be filed within the said period on separate sheets (Rule 36(1) EPC).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Article 96(3) EPC).



DEFFNER C A E
Primary Examiner
for the Examining Division

Enclosure(s): 2 page/s reasons (Form 2906)



The examination is being carried out on the following application documents:

Text for the Contracting States:

AT BE CH LI DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Description, pages:

1-64 as published

Claims, No.:

1-20 as received on 08.02.2002 with letter of 07.02.2002

Drawings, sheets:

1/10,3/10-10/19 as published

2/10 as received on 08.02.2002 with letter of 07.02.2002

- 1). The EST's cited in the international search report fall within the scope of present claim 1 (see entries N90606, geneseq, and M78230) Article 54 EPC).
- 2). Fragments defined in claim 1f) appear not to solve any problem unless they are capable of stimulating an immune response (page 46 3rd paragraph and page 3 2nd paragraph of the description) Article 56 EPC.
- 3). For remaining subject-matter the requirements of Articles 123(2), 54 and 56 EPC appear to be met.
- 4). Points 2 and 5 of the preceding communication should be respected.
- 5). To meet the requirements of Rule 27(1)(b) EPC, the documents disclosing the EST's cited in the international search report should be identified in the description and the relevant background art disclosed therein should be briefly discussed.



- 6). When filing amended claims the applicant should at the same time bring the description into conformity with the amended claims. Care should be taken during revision, especially of the introductory portion and any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Article 123(2) EPC).
- 7). In order to facilitate the examination of the conformity of the amended application with the requirements of Article 123(2) EPC, the applicant is requested to clearly identify the amendments carried out, irrespective of whether they concern amendments by addition, replacement or deletion, and to indicate the passages of the application as filed on which these amendments are based.

If the applicant regards it as appropriate these indications could be submitted in handwritten form on a copy of the relevant parts of the application as filed.

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Human Genome Sciences, Inc.
Our Ref.: C2276 EP

February 14, 2002
JAE/AD/ONG

This is in response to the Communication pursuant to Article 96 (2) EPC issued by the EPO on August 7, 2001 to the above identified case.

Please find enclosed new claims 1 to 20.

1. Amendments

1.1 New claim 1

New alternative (f) has been introduced finding support on page 26, second full paragraph of the description. Moreover, new alternative (g) has been incorporated. Support is to be found on page 34, first full paragraph. In new alternative (h), which is based on alternative (h) of pending claim 1, the term "having TNF delta or TNF epsilon activity" has been replaced by the term "capable of stimulating an immune response". Said measure is supported by the description on page 46, third paragraph. Alternative (f) and (g) of pending claim 1 have been deleted in new claim 1.

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1.2 New claims 2 to 10

New claims 2 to 10 correspond to pending claims 2 to 10 except that in claims 8 and 9, the term "having TNF delta or TNF epsilon activity" has been replaced by the term "capable of stimulating an immune response". Support for this measure is to be found as indicated under 1.1, supra.

1.3 New claim 11

New claim 11 is based on pending claim 11 except that the term "against" has been replaced by "specific for". Support for said measure is to be found in the description, e.g., on page 44 line 2, and lines 24 to 25.

1.4 New claim 12

New claim 12 corresponds to pending claim 12.

1.5 New claim 13

New claim 13 has been introduced instead of pending claim 13. Support for said claim is to be found on page 30, line 15, of the description.

1.6 New claims 14 to 20

New claims 14 to 20 correspond to pending claims 14 to 20.

1.7 Pending claim 21

Pending claim 21 has been deleted.

2 Inadmissible broadening (Article 123 (2) EPC) of pending and new claim 1

The Examining Division took the position that the polynucleotides comprised by alternative (f) of pending claim 1 and the fragments of at least 30 bases in length of alternative (g) of pending claim 1 lacked support by the application as originally filed.

Said objection, however, does not apply for new claim 1 as discussed in section 1.1, supra.

3 Clarity (Article 82 EPC)

3.1 Pending and new claim 11

The Examining Division took the position that pending claim 11 lacked clarity since due to its present working cross-reactive antibodies could also be comprised by said claim.

However, said objection does not apply to new claim 11 as discussed in section 1.3, supra. New claim 11 merely comprises those antibodies which specifically recognise the polypeptides of the present invention. Thus, the scope of new claim 11 is clear.

3.2 Pending and new claims 13 and 17

According to the Examining Division, the term "antagonists/inhibitor" as used in pending claim 13 or 17 would be unclear since said antagonists or inhibitors would not be defined by a technical feature and would therefore rather describe a result to be achieved.

However, said objection does not apply to new claims 13 and 17 as discussed in sections 1.5 and 1.6, supra.

In new claim 13, the antagonists and inhibitors have been structurally defined as being antibodies capable of inhibiting or extinguishing the activity of the polypeptides of the invention or as nucleic acid molecules capable of inhibiting the expression of the polynucleotides of the invention by binding thereto. Further, the person skilled in the art is well aware of how to determine whether an antibody is capable of inhibiting or extinguishing the activity of the polypeptides of the invention or whether a nucleic acid molecule is capable of inhibiting the expression of a polynucleotide of the invention.

In conclusion the antagonist/inhibitor of new claim 13 is clearly defined by technical features.

New claim 17 relates to the use of the antagonist / inhibitor of new claim 13 and is, therefore, also clear.

3.3 Pending claim 1 (h) and new claim 1 (g)

The Examining Division took the position that the TNF activity referred to the pending claim 1(h) would be unclear.

However, said objection does not apply for new claim 1(g) corresponding to pending claim 1(h); see section 1.1, supra. In new claim 1(g), the polynucleotides are defined as encoding a polypeptide being capable of stimulating an immune response. The term "stimulating an immune response" is clear for the person skilled in the art and described in detail on page 5, second paragraph and page 46, third paragraph. The person skilled in the art is well aware of how to determine if a protein is capable of stimulating an immune response. For example, a protein's ability to stimulate the activation and proliferation of immune cells (such as B cells and T cells) is indicative of said protein's ability to stimulate an immune response. Assays for measuring the activation and proliferation of immune cells are well known (see, e.g. Hahne et al (Annex A)).

Thus, the scope of new claim 1(g) is clear.

3.4 Pages 8 and 64

The Examining Division took the position that the last paragraphs on pages 8 and 64 would make the scope of the claims unclear.

However, we would defer any amendments of the description until the Examining Division has agreed on an allowable claims set.

4 **Inventive Step (Article 56 EPC)**

- 4.1 The Examining Division took the position that the claimed TNF variant polynucleotides and polypeptides lacked inventive step because they have been identified via their homology to already known TNF sequences. According to the Examining Division, the person skilled in the art could have identified the claimed sequences without any inventive activity.

We disagree.

For the assessment of inventive step it is not an issue whether the person skilled in the art could have arrived at the claimed solution of a technical problem, the issue is rather whether it would have done so (see, e.g., Guidelines, Chapter IV, part C, Section 9.5). In the following we will demonstrate by applying the technical problem-solution approach of the EPO that the present invention involves inventive step.

- 4.2 In the present case, the Examining Division apparently took the position that the available sequences for TNF α and TNF β referred to on page 4 could be seen as the closest prior art.
- 4.3 The technical problem underlying the present invention is the provision of means for regulating activation and differentiation of normal and abnormal cells as described on page 3, second paragraph of the description. Said technical problem is solved by the provision of polynucleotides and polypeptides encoding TNF delta and epsilon.
- 4.4 However, in light of the existing TNF α and TNF β sequences, there is no pointer towards the existence of further TNF family unless available in the prior art. Therefore, we take the position that it would not have been obvious to try to isolate new members of the TNF family based on homology screening.

However, even with the existence of TNF-alpha and TNF-beta, it would have been impossible to discover and isolate TNF-delta and/or TNF-epsilon. Experimental methods available at the time of filing for the cloning of new protein family members, require homology at the nucleotide level. If such homology does not exist, then one skilled in the art could not have isolated any new family members.

Attempts to align polynucleotides encoding TNF-alpha (Genbank Accession Number NM_000594, Annex B) or TNF-beta (Genbank Accession Number NM 000595, Annex C) with polynucleotides encoding TNF-delta (SEQ ID NO:1) or TNF-epsilon (SEQ ID NO:3) demonstrate that there is ***no significant homology*** between the polynucleotides encoding the prior art molecules, TNF-alpha and TNF-beta, and the polynucleotides encoding TNF-delta and TNF-epsilon (See Annexes D-G)¹. Therefore, one skilled in the art could not have isolated TNF-delta and/or TNF-epsilon using the nucleotide sequences disclosed in the prior art. Human Genome Sciences (HGS) identified the homology of TNF-delta and TNF-epsilon to TNF-alpha (and TNF-beta) only ***after*** HGS discovered the nucleotide sequence, translated the nucleotide sequence, and then aligned the sequence with known members of the TNF family.

Therefore, the person skilled in the art even when considering a homology based screen in order to arrive at the claimed solution of the technical problem would have had no reasonable expectation of success.

Consequently, inventive step must be acknowledged for the polynucleotides and polypeptides of the present invention.

¹ Note 1: Alignments were performed using the pairwise BLAST (Basic Local Alignment Search Tool) 2 Sequences algorithm available from the United States National Center for Biotechnology Information's website (<http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>) and described in Tanana A. Tamsova, Thomas L. Madden (1999), "Blast 2 sequences – a new tool for comparing protein and nucleotide sequences", FEMS Microbiol Lett. 174:247-250. As a "positive control", also attached as Annex H is an alignment between TNF-delta (SEQ ID NO:1) and TNF-epsilon (SEQ ID NO:3).

5. Request.

With the above explanations and the proposed modifications to the claims and the description, Applicant has met the requirements as set forth in the Official Communication.

If, however, the Examining Division does not agree to the above, it is requested that either a further Communication pursuant to Article 96(2) and Rule 51(2) EPC or a summons to attend oral proceedings according to Article 116(1) EPC be issued. If deemed expedient, an informal interview is requested. The undersigned is prepared to discuss minor amendments over the telephone.



Dr. Hans-Rainer Jaenichen
European Patent Attorney

Enclosure:

Annex A, Hahne et al.
~~Annex~~ B/C/D/E/F/G/H

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding a polypeptide comprising a fragment of at least 30 or at least 50 amino acids in length of a polynucleotide of any one of (a) to (d); and
 - (g) polynucleotides as defined in (f) that are operatively linked to a heterologous regulatory sequence.
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) and which encode a polypeptide capable of stimulating an immune response;
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

4. The polynucleotide of any one of claims 1 to 3 which is fused to a heterologous polynucleotide.
5. A vector containing the polynucleotide of any one of claims 1 to 4.
6. The vector of claim 5 in which the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
7. A host cell genetically engineered with the polynucleotide of any one of claims 1 to 4 or the vector of claim 5 or 6.
8. A process for producing a polypeptide capable of stimulating an immune response comprising: culturing the host cell of claim 7 and recovering the polypeptide encoded by said polynucleotide from the culture.
9. A process for producing cells capable of expressing a polypeptide which is capable of stimulating an immune response comprising genetically engineering cells with the vector of claim 5 or 6.
10. A polypeptide having the amino acid sequence encoded by a polynucleotide of any one of claims 1 to 4 or obtainable by the process of claim 8.
11. An antibody specific for the polypeptide of claim 10.
12. A nucleic acid molecule which specifically hybridizes to a polynucleotide of any one of claims 1 to 4.
13. An antagonist/inhibitor of the polypeptide of claim 10, wherein said antagonist/inhibitor is an antibody of claim 11 capable of inhibiting or extinguishing the activity of the polypeptide of claim 10 or a nucleic acid molecule of claim 12 capable of binding and thereby inhibiting the expression of the polynucleotide or the DNA of any one of claims 1 to 4.

14. A pharmaceutical composition comprising the polynucleotide of any one of claims 1 to 4, the polypeptide of claim 10 or a DNA encoding and capable of expressing said polypeptide in vivo or the antagonist/inhibitor of claim 13 and optionally a pharmaceutically acceptable carrier.
15. A diagnostic composition comprising the polynucleotide of any one of claims 1 to 4, the nucleic acid molecule of claim 12 or the antibody of claim 11.
16. Use of the polypeptide of claim 10 or the polynucleotide of any one of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment of neoplasia, for wound-healing, for the treatment of restenosis, for regulating hematopoiesis in endothelial cell development, for stimulating an immune response against parasitic, bacterial or viral infections, or for the treatment and/or prevention of autoimmune diseases.
17. Use of the antagonist/inhibitor of claim 13 for the preparation of a pharmaceutical composition for the treatment of cachexia, cerebral malaria, rheumatoid arthritis, for the prevention of graft-host rejection, for inhibiting bone resorption, for the treatment and/or prevention of osteoporosis, or for the treatment of endotoxic shock.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 10 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 10 in a sample derived from a host.
20. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 10 comprising:
 - (a) contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF

delta polypeptide and a compound under conditions to permit binding to the receptor; and

- (b) determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.

Brief Definitive Report

APRIL, a New Ligand of the Tumor Necrosis Factor Family, Stimulates Tumor Cell Growth

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Summary

Members of the tumor necrosis factor (TNF) family induce pleiotropic biological responses, including cell growth, differentiation, and even death. Here we describe a novel member of the TNF family designated APRIL (for a proliferation-inducing ligand). Although transcripts of APRIL are of low abundance in normal tissues, high levels of mRNA are detected in transformed cell lines, and in human cancers of colon, thyroid, and lymphoid tissues *in vivo*. The addition of recombinant APRIL to various tumor cells stimulates their proliferation. Moreover, APRIL-transfected NIH-3T3 cells show an increased rate of tumor growth in nude mice compared with the parental cell line. These findings suggest that APRIL may be implicated in the regulation of tumor cell growth.

Key words: tumor necrosis factor • tumorigenesis • cell survival • ligand • protein

Members of the TNF cytokine family are critically involved in the regulation of infections, inflammation, autoimmune diseases, and tissue homeostasis (1). These ligands can act in a membrane-bound form or as proteolytically processed, soluble cytokines in an autocrine, paracrine, or endocrine manner (1). Binding to their respective receptors leads to the triggering of diverse signaling pathways, including the activation of caspases, the translocation of nuclear factor- κ B (NF- κ B), or the activation of mitogen-activated kinases such as c-Jun NH₂-terminal kinase (JNK) or extracellular signal-regulatory kinase (ERK). Thus, TNF-related ligands can lead to either apoptosis, differentiation, or proliferation (1). To date, 13 members of the TNF cytokine family have been described: TNF- α , lymphotoxin (LT) α , LT β , CD40L, CD30L, CD27L, 4-1BBL, OX40L, FasL, TRAIL/APO-2L (2, 3), TRANCE/RANKL (4, 5), LIGHT (6), and TWEAK (7).

The ligand members are type II membrane molecules. Their extracellular domains have β jelly roll topography (8), and are important in ligand trimerization. Intrinsic to

oligomerization is the formation of the receptor binding site at the junction between neighboring subunits, creating a multivalent ligand. The binding of the ligands to their respective receptors induces oligomerization, initiating downstream signaling events.

Here we characterize the structural and functional properties of a new ligand of the TNF cytokine family. The new ligand, termed APRIL (for a proliferation-inducing ligand), is primarily expressed in tumor tissues and can accelerate the growth of transformed cells *in vitro* and *in vivo*.

Materials and Methods

Materials. The anti-FLAG M2 mAb and the anti-FLAG M2 antibody coupled to agarose were purchased from Eastman Kodak Co. (Rochester, NY). Protein A-Sepharose and CNBr-activated Sepharose were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Cell culture reagents were obtained from Life Sciences (Basel, Switzerland). Human TWEAK cDNA was provided by Dr. J. Browning (Biogen, Inc., Cambridge, MA). FLAG-tagged soluble human TWEAK (residues 141–284) and FLAG-tagged soluble FasL were produced in 293 cells as described (9, 10).

Cells. Murine B lymphoma A20 and human embryonic kidney cells 293 T (11) were maintained in DMEM containing 10%

T. Kataoka, M. Schröter, K. Hofmann, and M. Irmeler contributed equally to this work.

heat-inactivated FCS. The human T lymphoblastoma Jurkat cells, colon adenocarcinoma HT-29 cells, Raji Burkitt lymphoma, and human MCF-7 cells were grown in RPMI supplemented with 10% FCS. All media contained antibiotics (penicillin and streptomycin at 5 µg/ml each and neomycin at 10 µg/ml). Other cell lines referred to in this paper are deposited in and described by the American Type Culture Collection (Rockville, MD).

Northern Blot Analysis and In Situ Hybridization. Northern blot analysis was carried out using the following membranes: human multiple tissue Northern blots I and II (7760-1 and 7759-1; Clontech, Palo Alto, CA), human cancer cell line MTN blot (7757-1; Clontech), and human tumor panel blot V (D3500-01; Invitrogen Corp., Carlsbad, CA). The membranes were incubated in ExpressHyb hybridization solution (8015-1; Clontech) for at least 1 h at 62°C. The random-primed cDNA probe (Boehringer Mannheim Corp., Indianapolis, IN) was synthesized using cDNA corresponding to the extracellular domain of APRIL as template. The heat-denatured cDNA probe was added at 1.5×10^6 cpm/ml in fresh ExpressHyb. The membrane was hybridized 12–24 h at 62°C, washed three times in $2\times$ SSC containing 0.05% SDS, and exposed at -70°C to x-ray films.

For in situ hybridization, cryostat sections (6–8 µm) of primary colon carcinomas (three patients) were air dried, fixed in 4% (wt/vol) paraformaldehyde in PBS, and used immediately for immunofluorescence or stored in 70% ethanol at 4°C before in situ hybridization. In situ hybridization and immunofluorescence were performed as reported previously (12). After in situ hybridization, slides were directly exposed to x-ray films. Specificity controls included the systematic use of sense cRNA probes in each experiment.

Characterization of APRIL cDNA and Expression of Recombinant Soluble APRIL. The full-length APRIL gene (sequence data available from EMBL/GenBank/DBJ under accession no. AF046888) was contained in the expressed sequence tag (EST) clones AA292304 and AA292358. EST clone AA292304 was used to amplify the coding region of APRIL using a specific 5' forward primer flanked by an EcoRI site (5'-CCAGCCTCATCTCCTTTCTTGC-3') and a specific 3' reverse primer flanked by an XbaI site (5'-TCACAGTTTCACAAACCCAGG-3'). The amplified fragment was cut with EcoRI/XbaI and cloned into a modified version of pCRII (Invitrogen Corp.), in frame with an NH₂-terminal FLAG peptide (9). The soluble form of APRIL (sAPRIL) was generated using the two primers 5'-AAACAGAA-GAAGCAGCACTCTG-3' and 5'-TCACAGTTTCACAAACCCAGG-3' containing a PstI and XbaI site, respectively, and subsequently cloned into a modified pCRII vector containing both a hemagglutinin signal for protein secretion in eukaryotic cells and an NH₂-terminal FLAG epitope (9). Purification of FLAG-tagged APRIL was affinity-purified on anti-FLAG M2 antibody coupled to agarose.

Proliferation Assays. The proliferation of cells was determined by incubating 5×10^4 cells/well in 100 µl medium with the indicated concentrations of recombinant sAPRIL, sTWEAK, and sFasL using the Celltiter 96 AQ proliferation assay (Promega Corp., Madison, WI) after 24 h, following the manufacturer's instructions. Alternatively, cells were pulsed for 4 h with [³H]thymidine (0.5 µCi/well), exposed to three cycles of freezing and thawing, and harvested. [³H]Thymidine incorporation was monitored by liquid scintillation counting. For the immunodepletion of FLAG-APRIL, anti-FLAG antibodies coupled to agarose (Eastman Kodak Co.) were used.

Cell Lines Stably Expressing APRIL. The full-length FLAG-tagged APRIL containing pCRII expression vector was trans-

fected into NIH-3T3 cells using the calcium phosphate method of transfection. 48 h after transfection, cells were seeded at 10^4 cells/well in flat-bottomed 96-well plates under selection with 800 µg/ml G418 (Sigma Chemical Co., St. Louis, MO). Cell extracts of stable clones were electrophoretically separated by SDS-PAGE under reducing conditions and subsequently transferred to nitrocellulose. Immunoblots of FLAG-tagged APRIL were probed using 5 µg/ml of anti-FLAG M2 mAb and the ECL system (Amersham Pharmacia Biotech).

Tumor Growth in Mice. NIH-3T3 fibroblasts (American Type Culture Collection) and the various transfectants (10^5 cells) were suspended in 50 µl PBS and injected subcutaneously into the flank region of BALB/c nude mice (Harlan Nederland, Zeist, The Netherlands). Mice were sex- and age-matched.

Results and Discussion

APRIL Is a Novel Ligand of the TNF Family. All TNF ligand/receptor family members characterized to date are biologically important. Therefore, we screened public databases using an improved profile search (13) based on an optimal alignment of all currently known TNF ligand family members. Several candidate clones were found coding for a unique, novel TNF-α-related ligand which we termed APRIL (for a proliferation-inducing ligand). Two of these cDNA clones (AA292358 and AA292304) contained full-length sequences (1.5 and 1.7 kb, respectively) encoding a protein of 250 amino acids, with a predicted cytoplasmic domain of 28 amino acids, a hydrophobic transmembrane region, and an extracellular domain of 201 amino acids (Fig. 1 A). The absence of a signal peptide suggested that APRIL was a type II membrane protein which is typical of the members of the TNF ligand family. The single N-linked glycosylation site (N124) predicted for this protein lies within the first of several β strands which are folded into an antiparallel β sandwich structure (14). The sequence of the extracellular domain of APRIL showed highest homology with FasL (21% amino acid identity), TNF-α (20%), and LTβ (18%), followed by TRAIL, TWEAK, and TRANCE (15%) (Fig. 1 B).

Expression of APRIL mRNA in Tumors. Northern blot analysis of APRIL revealed that the expression of APRIL was weak and restricted only to a few tissues (Fig. 2 A). Two transcripts of 2.1 and 2.4 kb were found in the prostate, whereas a shorter 1.8-kb transcript was found in PBLs. A longer exposure time of the Northern blot revealed that the 2.1-kb APRIL mRNA was also present in colon, spleen, and pancreas (data not shown). This restricted distribution of the APRIL mRNA was consistent with the origin of cDNA clones currently available in the EST database. Of the 23 clones identified, only two were derived from normal tissues (pregnant uterus and pancreatic islet cells). Remarkably, the remainder of the EST clones (21 clones, 91%) were present in cDNA libraries generated from tumors or tumor-derived cell lines (ovary tumor, 11; prostate tumor, 3; Gessler Wilms tumor, 1; colon carcinoma, 1; endometrial tumor, 1; parathyroid tumors, 1; pancreas tumor, 1; T cell lymphoma, 1; LNCAP adenocar-

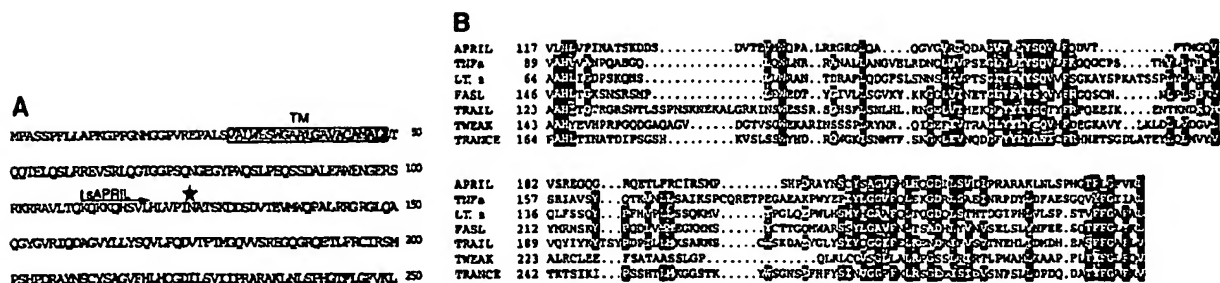


Figure 1. (A) Predicted amino acid sequence of human APRIL. The predicted transmembrane region (TM, boxed), the potential N-linked glycosylation site (*), and the NH₂ terminus of the recombinant sAPRIL are indicated. (B) Comparison of the extracellular protein sequence of APRIL and some members of the TNF ligand family. Identical and homologous residues are represented in black and shaded boxes, respectively. TNFa, TNF- α ; LT α , LT α .

cinoma-derived cell line, 1). This is in contrast to TNF- α , where only 16% of EST clones were tumor-derived. This prompted us to test transformed cell lines for the expression of APRIL mRNA (Fig. 2 B), and indeed, all tumor cell lines strongly expressed the 2.1-kb transcript of APRIL. The highest APRIL-specific signals were detected in the colorectal adenocarcinoma SW480, the Burkitt's lymphoma Raji, and the melanoma G361 cell lines.

To corroborate this finding, we measured APRIL mRNA expression levels in several tumors and compared them to normal tissues. APRIL mRNA was elevated in thyroid carcinoma and in lymphoma, whereas in the corresponding normal tissues, hybridization signals were either

weak or absent (Fig. 2 C). In the two other tumors analyzed by Northern blots (adrenal and parotid carcinoma), APRIL mRNA was not increased. By in situ hybridization, highly increased levels of APRIL mRNA were also detected in human colon adenocarcinomas of three different patients compared with normal colon tissue (Fig. 2 D).

APRIL Enhances Tumor Cell Proliferation. To explore the possible activities of APRIL, we expressed a FLAG-tagged form of the soluble extracellular domain of APRIL (sAPRIL) encompassing amino acids 110–250 in 293 cells. The widespread expression of APRIL in tumor cells and tissues suggested to us that APRIL may be associated with tumor growth, and we therefore incubated various tumor



Figure 2. Expression of APRIL. (A) Northern blots (2 μ g polyA⁺ RNA/lane) of various human tissues were probed with APRIL cDNA. (B) APRIL mRNA expression in various tumor cell lines: promyelocytic leukemia HL60; HeLa cell S3; chronic myelogenous leukemia K562; lymphoblastic leukemia Molt-4; Raji Burkitt's lymphoma; colorectal adenocarcinoma SW480; lung carcinoma A459; melanoma and G361. (C) APRIL mRNA expression in four different human tumors (T) and normal tissues (N). The 18S rRNA band shows equal loading. (D) APRIL mRNA expression in primary colon carcinoma. In situ hybridization reveals abundant APRIL message in human colon carcinoma. Colon tumor tissue sections and adjacent normal tissue were hybridized to antisense APRIL ³²S-labeled cRNA, and as control, colon tumor tissue sections were also hybridized to sense APRIL ³²S cRNA (negative control). Top, Dark field micrographs; bottom, the corresponding light field micrographs.

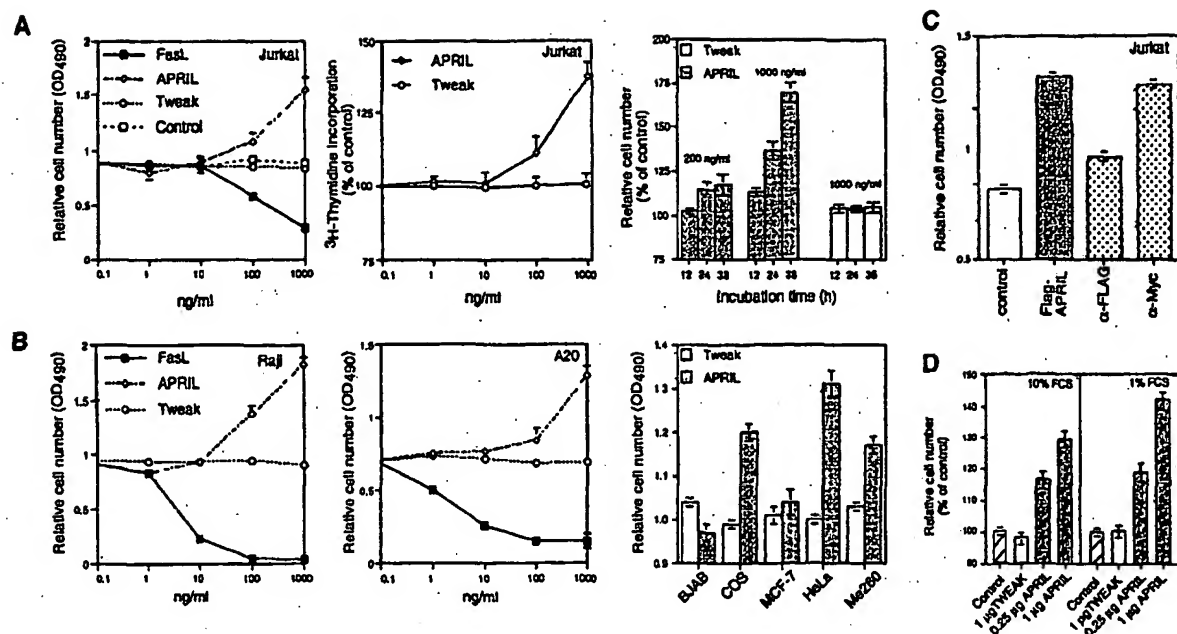


Figure 3. APRIL stimulates cell growth. (A) Dose-dependent stimulation of proliferation of Jurkat cells (human leukemic T cells), determined 24 h after the addition of sAPRIL. Controls include cells treated with FasL, TWEAK, and no ligand (Control). Left, Number of viable cells; middle, [³H]thymidine incorporation; right, kinetic analysis of the effect of APRIL on Jurkat cells. The concentrations of ligands are indicated. (B) Effect of APRIL on the proliferation rate of Raji (human Burkitt lymphoma), A20 (mouse B lymphoma), BJAB (human B lymphoma), COS (SV40-transformed monkey kidney cells), MCF-7 (human breast adenocarcinoma), HeLa (human embryonic lung cell), and ME260 (human melanoma). (C) Influence of immunodepletion of FLAG-tagged APRIL on tumor cell growth. The proliferative effect of FLAG-tagged APRIL is neutralized by Sepharose-bound anti-FLAG antibodies, but not by anti-myc antibodies. (D) Influence of FCS concentration on APRIL-induced proliferation of Jurkat cells. Data are the means \pm SEM of triplicate determinations.

cell lines with purified recombinant sAPRIL. An increase in proliferation of the Jurkat T lymphoma cells in the presence of APRIL was observed in a dose-dependent manner as detected by an increase in the number ($\sim 50\%$) of viable cells 24 h after ligand addition (Fig. 3 A). As expected, the addition of identically produced and purified FasL to Jurkat cells decreased the number of viable cells, whereas TWEAK had no effect. The increased cell number correlated with augmented (40%) [³H]thymidine incorporation in APRIL-treated cells (Fig. 3 A). Differences in growth rates of Jurkat cells exposed to APRIL and TWEAK were already apparent after 12 h of incubation, and after 36 h the number of Jurkat cells had almost doubled (Fig. 3 A, right). Immunodepletion of FLAG-tagged APRIL-containing medium by anti-FLAG antibodies, but not anti-myc antibodies, reduced the proliferative effect (Fig. 3 C), indicating that the stimulation of proliferation was specific and due to the presence of APRIL. Increased rates in proliferation were also seen in some B lymphomas (human Raji cells, mouse A20 cells, but not human BJAB cells) and in cell lines of epithelial origin, such as COS and HeLa, as well as melanomas (Fig. 3 B). The breast carcinoma cell MCF-7 did not respond. The effect on Jurkat cells was even more intense when the concentration of FCS was reduced from 10 to 1% (Fig. 3 D).

Tumor Cells Expressing APRIL Display an Increased Growth Rate in Mice. We next transfected NIH-3T3 cells with full-length human APRIL and obtained several APRIL-expressing clones (Fig. 4 A). Interestingly, APRIL transfect-

ants proliferated faster than mock-transfected cells or wild-type cells (Fig. 4 B). Consequently, we asked the question whether the APRIL-transfected NIH-3T3 cells might also have a growth advantage in vivo. When wild-type or mock-transfected NIH-3T3 cells were injected into nude mice, small palpable tumors were observed after 5–6 wk. In contrast, the two clones of NIH-3T3 cells stably transfected with APRIL which were injected into nude mice both induced tumors after only 3–4 wk. After 6 wk, mice had to be killed due to the high tumor burden (Fig. 4 C).

Many of the TNF-related ligands can induce pleiotropic biological responses. Ligands trigger costimulatory signals in most cases, induce cell death, or lead to proliferation of primary cells. For instance, TNF-R2 triggering leads to proliferation of thymocytes (15), and the biological function of CD40-40L provides a clear example of costimulation (14). TNF- α was discovered as a cytokine with the capacity to induce tumor necrosis (16). Unlike these ligands, APRIL can stimulate the growth of tumor cell lines. APRIL also appears to be unique among the TNF family, as it is abundantly expressed in tumor cells. Collectively, these results suggest that the physiological function(s) of APRIL is distinct from other members of the TNF family. Preliminary results indicate that the APRIL gene is not linked to the TNF/LT locus in the MHC.

The mechanism by which APRIL increases cellular proliferation of transformed cells is currently unknown. APRIL does not appear to activate NF- κ B or c-Jun NH₂-terminal

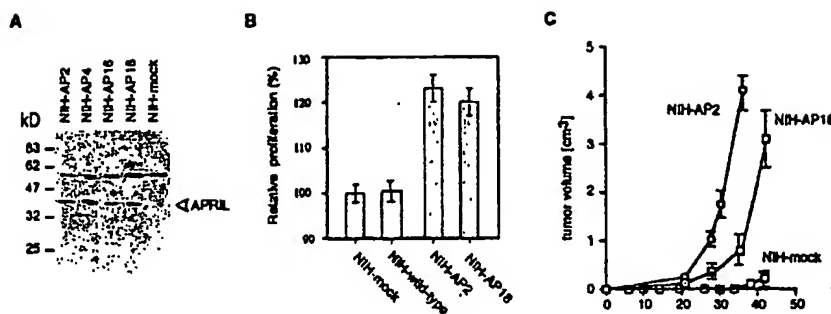


Figure 4. APRIL accelerates tumor growth. (A) Characterization of APRIL-transfected NIH-3T3 clones. FLAG-APRIL levels of the various clones were analyzed by Western blotting using an anti-FLAG antibody. Arrow, The APRIL protein; the high molecular weight protein is detected non-specifically. (B) APRIL-expressing NIH-3T3 clones grow faster than mock-transfected clones. (C) Increased tumor growth of APRIL-expressing NIH-3T3 clones. NIH-3T3 cells (10^5 cells) and APRIL (NIH-AP, two different clones) transfectants (10^5 cells) were injected subcutaneously into nude mice, and tumor growth was monitored. Data are representative of three experiments with six mice per group.

kinase, and the cell cycle analysis of APRIL-treated cells remains unperturbed (data not shown). Moreover, the histochemical analysis of tumors induced by APRIL-transfected NIH-3T3 cells did not reveal any apparent morphological differences compared with wild-type NIH-3T3 tumors, suggesting that APRIL is not angiogenic. Thus, it will be important to identify and characterize the receptor for APRIL (recombinant sAPRIL does not interact with Fas, LT β R, HVEM, TNF-R1, TNF-R2, GITR, TRAIL-R1, TRAIL-R2, or TRAIL-R3; data not shown).

The development of cancer is viewed as a multistep pro-

cess, involving mutation and selection for cells with progressively increasing capacity for proliferation, survival, and invasion, even under conditions where the growth factor (blood) supply is limited. APRIL allows tumor cells to proliferate at a reasonable rate even in low serum conditions (see Fig. 3 D). Given that APRIL is strongly expressed in several types of tumors and that it stimulates cell proliferation in vitro and in vivo, it is possible that APRIL may play a role in tumorigenesis. Therefore, antagonistic antibodies to APRIL or the APRIL receptor may have a potential for cancer treatment.

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10. Schneider, P., N. Holler, J.L. Bodmer, M. Hahne, K. Frei, A. Fontana, and J. Tschopp. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J. Exp. Med.* 187:1205-1213.
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12. French, L.E., M. Hahne, I. Viard, G. Radgruber, R. Zanone, K. Becker, C. Muller, and J. Tschopp. 1996. Fas and Fas ligand in embryos and adult mice: ligand expression in several immune-privileged tissues and coexpression in adult tissues characterized by apoptotic cell turnover. *J. Cell Biol.* 133:335-343.
13. Bucher, P., K. Karplus, N. Moeri, and K. Hofmann. 1996. A flexible search technique based on generalized profiles. *Comput. Chem.* 20:3-23.
14. Banner, D.W., A. D'Arcy, W. Janes, R. Gentz, H.J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell*. 73:431-445.
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16. Tracey, K.J., and A. Cerami. 1994. Tumor necrosis factor: a pleiotropic cytokine and therapeutic target. *Annu. Rev. Med.* 45:491-503.

Annex B

☐ 1: NM_000594. Homo sapiens tumor necrosis factor (TNF) superfamily, member 2 (gi:10835154)

LOCUS NM_000594 1585 bp mRNA linear PRI 31-OCT-2000
DEFINITION Homo sapiens tumor necrosis factor (TNF) superfamily, member 2 (TNF), mRNA.
ACCESSION NM_000594
VERSION NM_000594.1 GI:10835154
KEYWORDS
SOURCE human.
ORGANISM Homo sapiens
 Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.

REFERENCE 1 (bases 1 to 1585)
AUTHORS Pennica D, Nedwin GE, Hayflick JS, Seeburg PH, Derynck R, Palladino MA, Kohr WJ, Aggarwal BB and Goeddel DV.
TITLE Human tumour necrosis factor: precursor structure, expression and homology to lymphotoxin
JOURNAL Nature 312 (5996), 724-729 (1984)
MEDLINE 85086244
PUBMED 6392892

REFERENCE 2 (bases 1 to 1585)
AUTHORS Shirai T, Yamaguchi H, Ito H, Todd CW and Wallace RB.
TITLE Cloning and expression in Escherichia coli of the gene for human tumour necrosis factor
JOURNAL Nature 313 (6005), 803-806 (1985)
MEDLINE 85137898
PUBMED 3883195

REFERENCE 3 (bases 1 to 1585)
AUTHORS Wang, A.M., Creasey, A.A., Lachner, M.B., Lin, L.S., Strickler, J., Van Arsdel, J.N., Yamamoto, R. and Mark, D.F.
TITLE Molecular cloning of the complementary DNA for human tumor necrosis factor
JOURNAL Science 228 (4696), 149-154 (1985)
MEDLINE 85142190

REFERENCE 4 (bases 1 to 1585)
AUTHORS Nedwin GE, Naylor SL, Sakaguchi AY, Smith D, Jarrett-Nedwin J, Pennica D, Goeddel DV and Gray PW.
TITLE Human lymphotoxin and tumor necrosis factor genes: structure, homology and chromosomal localization
JOURNAL Nucleic Acids Res. 13 (17), 6361-6373 (1985)
MEDLINE 86016093
PUBMED 2995927

REFERENCE 5 (bases 1 to 1585)
AUTHORS Marmenout A, Franssen L, Tavernier J, Van der Heyden J, Tizard R, Kawashima E, Shaw A, Johnson MJ, Semon D, Muller R and et al.
TITLE Molecular cloning and expression of human tumor necrosis factor and comparison with mouse tumor necrosis factor
JOURNAL Eur. J. Biochem. 152 (3), 515-522 (1985)
MEDLINE 86030296
PUBMED 3932069

REFERENCE 6 (bases 1 to 1585)
AUTHORS Old LJ.
TITLE Tumor necrosis factor (TNF)
JOURNAL Science 230 (4726), 630-632 (1985)
MEDLINE 86018867

PUBMED 2413547
 REFERENCE 7 (bases 1 to 1585)
 AUTHORS Nedospasov SA, Shakhov AN, Turetskaya RL, Metc VA, Azizov MM, Georgiev GP, Kocobko VG, Dobrynin VN, Filippov SA, Bystrov NS and et al.
 TITLE Tandem arrangement of genes coding for tumor necrosis factor (TNF-alpha) and lymphotoxin (TNF-beta) in the human genome
 JOURNAL Cold Spring Harb. Symp. Quant. Biol. 51 Pt 1. 611-624 (1986)
 MEDLINE 87217060
 PUBMED 3555974
 REFERENCE 8 (bases 1 to 1585)
 AUTHORS Davis JM, Narachi MA, Alton NK and Arakawa T.
 TITLE Structure of human tumor necrosis factor alpha derived from recombinant DNA
 JOURNAL Biochemistry 26 (5). 1322-1326 (1987)
 MEDLINE 87185436
 PUBMED 3552045
 COMMENT PROVISIONAL REFSEQ: This record has not yet been subject to final NCBI review. The reference sequence was derived from M10988.1.
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Revised: October 24, 2001.

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Annex C

☐ 1: NM_000595. Homo sapiens lymph...[gi:6806892]

[Related Sequences](#), [OMIM](#), [Protein](#), [PubMed](#), [Taxonomy](#), [UniSTS](#), [LinkOut](#)

LOCUS NM_000595 1386 bp mRNA linear PRI 22-FEB-2001
DEFINITION Homo sapiens lymphotoxin alpha (TNF superfamily, member 1) (LTA), mRNA.
ACCESSION NM_000595
VERSION NM_000595.2 GI:6806892
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SOURCE human.
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;
Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 1386)
AUTHORS Aggarwal, S.S., Moffat, B. and Harkins, R.N.
TITLE Human lymphotoxin. Production by a lymphoblastoid cell line.
purification, and initial characterization
JOURNAL The Journal of biological chemistry. 259 (1), 626-631 (1984)
MEDLINE 84161986
PUBMED 6608523
REFERENCE 2 (bases 1 to 1386)
AUTHORS Gray, P.W., Aggarwal, B.B., Benton, C.V., Bringman, T.S., Henzel, W.J.,
Jarrett, J.A., Leung, D.W., Moffat, B., Ng, P., Svedersky, L.P.,
Palladino, M.A. and Nedwin, G.E.
TITLE Cloning and expression of cDNA for human lymphotoxin, a lymphokine
with tumour necrosis activity
JOURNAL Nature. 312 (5996), 721-724 (1984)
MEDLINE 85086243
PUBMED 6334807
REFERENCE 3 (bases 1 to 1386)
AUTHORS Nedwin, G.E., Naylor, S.L., Sakaguchi, A.Y., Smith, D.,
Jarrett-Nedwin, J., Pennica, D., Goeddel, D.V. and Gray, P.W.
TITLE Human lymphotoxin and tumor necrosis factor genes: structure,
homology and chromosomal localization
JOURNAL Nucleic acids research. 13 (17), 6361-6373 (1985)
MEDLINE 86016093
PUBMED 2995927
REFERENCE 4 (bases 1 to 1386)
AUTHORS Aggarwal, B.B., Zessalu, T.Z. and Hass, P.S.
TITLE Characterization of receptors for human tumour necrosis factor and
their regulation by gamma-interferon
JOURNAL Nature. 318 (6047), 565-567 (1985)
MEDLINE 86092209
PUBMED 3001529
REFERENCE 5 (bases 1 to 1386)
AUTHORS Kobayashi, Y., Miyamoto, D., Asada, M., Obinata, M. and Osawa, T.
TITLE Cloning and expression of human lymphotoxin mRNA derived from a
human T cell hybridoma
JOURNAL Journal of biochemistry. 100 (3), 727-733 (1986)
MEDLINE 87057135
PUBMED 3536896
REFERENCE 6 (bases 1 to 1386)
AUTHORS Nedospasov SA, Shakhov AN, Turatskaya AL, Mett VA, Azizov MM,
Georgiev GP, Korobko VG, Dobrynin VN, Filippov SA, Bystrov NS and
et al.
TITLE Tandem arrangement of genes coding for tumor necrosis factor

(TNF-alpha) and lymphotoxin (TNF-beta) in the human genome
Cold Spring Harbor symposia on quantitative biology. 51 Pt 1.
611-624 (1986)

JOURNAL
MEDLINE 87217050
PUBMED 3555974
REFERENCE 7 (bases 1 to 1386)
AUTHORS Evans, A.M., Petersen, J.W., Sekhon, G.S. and Demars, R.
TITLE Mapping of prolactin and tumor necrosis factor-beta genes on human chromosome 6p using lymphoblastoid cell deletion mutants
Somatic cell and molecular genetics. 15 (3), 203-213 (1989)

JOURNAL
MEDLINE 89267210
PUBMED 2567039
REFERENCE 8 (bases 1 to 1386)
AUTHORS Messer, G., Spengler, U., Jung, M.C., Honold, G., Blomer, K., Pape, G.R., Riechmüller, G. and Weiss, E.H.
TITLE Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human TNF-beta gene correlates with a variant amino acid in position 26 and a reduced level of TNF-beta production
The Journal of experimental medicine. 173 (1), 209-219 (1991)

JOURNAL
MEDLINE 91086845
PUBMED 1670638
REFERENCE 9 (bases 1 to 1386)
AUTHORS Abraham, L.J., Du, D.C., Zahedi, K., Dawkins, R.L. and Whitehead, A.S.
TITLE Haplotypic polymorphisms of the TNFB gene
Immunogenetics. 33 (1), 50-53 (1991)

JOURNAL
MEDLINE 91139175
PUBMED 1671667
REFERENCE 10 (bases 1 to 1386)
AUTHORS Matsuyama, N., Okawa, N., Tsukitani, Y., Endo, T. and Kaji, A.
TITLE Nucleotide sequence of a cDNA encoding human tumor necrosis factor beta from B lymphoblastoid cell RPMI 1788
FEBS letters. 302 (2), 141-144 (1992)

JOURNAL
MEDLINE 92339500
PUBMED 1353024
REFERENCE 11 (bases 1 to 1386)
AUTHORS Iris FJ, Bouguérierat L, Prieur S, Caterina D, Primas G, Perrot V, Jurka J, Rodriguez-Tome P, Claverie JM, Dausset J and et al.
TITLE Dense Alu clustering and a potential new member of the NF kappa B family within a 90 kilobase HLA class III segment
Nature genetics. 3 (2), 137-145 (1993)

JOURNAL
MEDLINE 93272029
PUBMED 8499947
REFERENCE 12 (bases 1 to 1386)
AUTHORS Browning, J.L., Ngam-ek, A., Lawton, P., Demarinis, J., Tizard, R., Chow, E.P., Messian, C., O'Brine-Graco, E., Foley, S.F. and Ware, C.F.
TITLE Lymphotoxin beta, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface
Cell. 72 (6), 847-856 (1993)

JOURNAL
MEDLINE 93208881
PUBMED 7916655
REFERENCE 13 (bases 1 to 1386)
AUTHORS Albertella, M.R. and Campbell, R.D.
TITLE Characterization of a novel gene in the human major histocompatibility complex that encodes a potential new member of the I kappa B family of proteins
Human molecular genetics. 3 (5), 793-799 (1994)

JOURNAL
MEDLINE 94362579
PUBMED 8081366
REFERENCE 14 (bases 1 to 1386)
AUTHORS Warocha, K., Bienvenu, J., Coiffier, B. and Salles, C.
TITLE Mechanisms of action of the tumor necrosis factor and lymphotoxin ligand-receptor system
Eur. Cytokine Netw. 5 (6), 83-96 (1994)

JOURNAL
REFERENCE 15 (bases 1 to 1386)
AUTHORS Holzinger, I., de Baey, A., Messer, G., Kick, G., Zwierrina, H. and Weiss, E.H.

TITLE Cloning and genomic characterization of LST1: a new gene in the human TNF region
 JOURNAL Immunogenetics. 42 (5), 315-322 (1995)
 MEDLINE 96006565
 PUBMED 7590964
 REFERENCE 16 (bases 1 to 1386)
 AUTHORS Peelman LJ, Chardon P, Nunes M, Renard C, Geffroy C, Vaiman M, Van Zeven A, Coppieters W, van de Weghe A, Bouquet Y and et al.

TITLE The BAT1 gene in the MHC encodes an evolutionarily conserved putative nuclear RNA helicase of the DEAD family
 JOURNAL Genomics. 26 (2), 210-218 (1995)
 MEDLINE 95324911
 PUBMED 7601445
 REFERENCE 17 (bases 1 to 1386)
 AUTHORS Urans, U., Quist, W.C., McManus, B.M., Wilson, J.E., Arceci, R.J., Wallace, A.F. and Russell, M.E.

TITLE Allograft inflammatory factor-1. A cytokine-responsive macrophage molecule expressed in transplanted human hearts
 JOURNAL Transplantation. 61 (9), 1387-1392 (1996)
 MEDLINE 96215741
 PUBMED 8629302
 REFERENCE 18 (bases 1 to 1386)
 AUTHORS Korner, H. and Sedgwick, J.D.

TITLE Tumour necrosis factor and lymphotoxin: molecular aspects and role in tissue-specific autoimmunity
 JOURNAL Immunology and cell biology. 74 (5), 465-472 (1996)
 MEDLINE 97068822
 PUBMED 8912010
 REFERENCE 19 (bases 1 to 1386)
 AUTHORS de Baey, A., Fellerhoff, B., Maier, S., Martinozzi, S., Weidle, U. and Weiss, E.H.

TITLE Complex expression pattern of the TNF region gene LST1 through differential regulation, initiation, and alternative splicing
 JOURNAL Genomics. 45 (3), 591-600 (1997)
 MEDLINE 98035883
 PUBMED 9367684
 REFERENCE 20 (bases 1 to 1386)
 AUTHORS Neville, M.J. and Campbell, R.D.

TITLE Alternative splicing of the LST-1 gene located in the Major Histocompatibility Complex on human chromosome 6
 JOURNAL DNA sequence : the journal of DNA sequencing and mapping. 8 (3), 155-160 (1997)
 MEDLINE 20132445
 PUBMED 10668961
 REFERENCE 21 (bases 1 to 1386)
 AUTHORS Shiina T, Tamiya G, Oka A, Yamagata T, Yamagata N, Kikkawa E, Goto K, Mizuki N, Watanabe K, Fukuzumi Y, Taguchi S, Sugawara C, Ono A, Chen L, Yamazaki M, Tashiro H, Ando A, Ikemura T, Kimura M and Inoko H.

TITLE Nucleotide sequencing analysis of the 146-kilobase segment around the IkBL and MICA genes at the centromeric end of the HLA class I region
 JOURNAL Genomics. 47 (3), 372-382 (1998)
 MEDLINE 98149985
 PUBMED 9480751

COMMENT REVIEWED REFSEQ: This record has been curated by NCBI staff. The reference sequence was derived from D12614.1, M55913.1. On Jan 28, 2000 this sequence version replaced gi:4505030.
 Summary: Lymphotoxin alpha, a member of the tumor necrosis factor family, is a cytokine produced by lymphocytes. LTA is highly inducible, secreted, and exists as homotrimeric molecule. LTA forms heterotrimers with lymphotoxin-beta which anchors lymphotoxin-alpha to the cell surface. LTA mediates a large variety of inflammatory, immunostimulatory, and antiviral responses. LTA is also involved in the formation of secondary lymphoid organs during development and plays a role in apoptosis. COMPLETENESS: complete on the 3' end.

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1141 ttattctatt atggaggatg gaggagaggg aataatagaa gaacatccaa ggagaaacag
1201 agacaggccc aagagatgaa gagtgaaggg gcatgcgcac aaggctgacc aagagagada
1261 gaagtaggca tgaggatca cagggcccca gaaggcaggg aaaggctctg aaagccagct
1321 gccgaccaga gcccacacg gaggcatctg caccctcgat gaagcccaat aaacctctct
1381 tctctg

```

//

Revised: October 24, 2001

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Annex D



Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

Match: Mismatch: gap open: gap extension:
x_dropoff: expect: wordsize: ☒ Filter ☐ Align

Sequence 1 lc|seq_1 Length 1585

TNF-alpha (Genbank MM_000594)

Sequence 2 lc|seq_2 Length 1717

TNF-delta (SEQ ID NO:1)

No significant similarity was found

Annex E



Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

Match: Mismatch: gap open: gap extension:
lambda_dropoff: expect: wordsize: ☒ Filter ☒ Align

Sequence 1 lc|seq_1 Length 1585

TNF-alpha (Genbank NM_000594)

Sequence 2 lc|seq_2 Length 1281

TNF-epsilon (SEQ ID NO:3)

No significant similarity was found

Annex F



Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

Match: Mismatch: gap open: gap extension:
x_dropoff: expect: wordsize: ☐ Filter ☒ Align

Sequence 1 lc|seq_1 Length 1386 TNF-beta (Genbank NM_000595)

Sequence 2 lc|seq_2 Length 1717 TNF-delta (SEQ ID NO.1)

No significant similarity was found

Annex G



Blast 2 Sequences results

[PubMed](#)[Entrez](#)[BLAST](#)[OMIM](#)[Taxonomy](#)[Structure](#)

BLAST 2 SEQUENCES RESULTS VERSION BLASTN 2.1.2 [Oct-19-2000]

Match: Mismatch: gap open: gap extension:
x_dropoff: expect: wordsize: Filter ☒ Align

Sequence 1 |cl|seq_1 Length 1386 TNF-beta (Genbank MM_000595)

Sequence 2 |cl|seq_2 Length 1281 TNF-epsilon (SEQ ID NO:3)

No significant similarity was found

Annex H



Entrez

BLAST

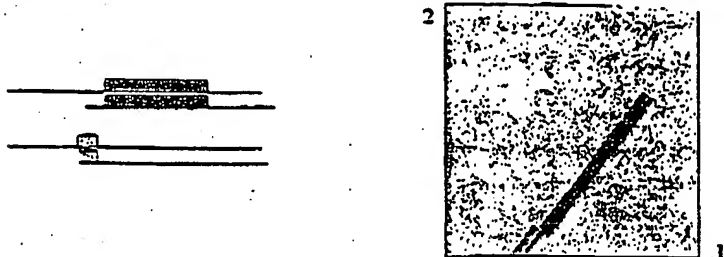
OMIM.

Taxonomy

Structure

Match: 1 Mismatch: -2 gap open: 5 gap extension: 2
x_dropoff: 20 expect: 10.0 wordsize: 11 Filter 2 Align

Sequence 1 |cl|seq_1 Length 1717 (1 . 1717) TNF-delta (SEQ ID NO:1)
Sequence 2 |cl|seq_2 Length 1281 (1 . 1281) TNF-epsilon (SEQ ID NO:3)



Score = 1296 bits (674).. Expect = 0.0
Identities = 687/691 (99%).. Gaps = 1/691 (0%)
Strand = Plus / Plus

Query: 663	aaggatgactccgatagtgacagagggatgctgggcaaccagctctctaggcgtgggagagggc	722
Subject: 137	aaagaatgactccgatagtgacagagggatgctgggcaaccagctctctaggcgtgggagagggc	196
Query: 723	ctacagggcccaaggatcatgggtgtccgaattccaggatgctggagctcttatctgctgtctacagc	782
Subject: 197	ctacagggcccaaggatcatgggtgtccgaattccaggatgctggagctcttatctgctgtctacagc	256
Query: 783	cagggtccctggttcaagacgtgactttcacccatgggtcagggtgggtgtctctcgagaaggccaa	842
Subject: 257	cagggtccctggttcaagacgtgactttcacccatgggtcagggtgggtgtctctcgagaaggccaa	316
Query: 843	ggaaggcaggagacttctatctccgactgataaagaagtatgcccccccacccggaccggggcc	902
Subject: 317	ggaaggcaggagacttctatctccgactgataaagaagtatgcccccccacccggaccggggcc	376
Query: 903	tacaacagctgctatagcgcagggtgtcttccattacaccaaggggatattctcgagtgtc	962
Subject: 377	tacaacagctgctatagcgcagggtgtcttccattacaccaaggggatattctcgagtgtc	436
Query: 963	ataaatccccgggcaaggggcgaaacttaacctctctccacatgggaaccttccctgggggtct	1022
Subject: 437	ataaatccccgggcaaggggcgaaacttaacctctctccacatgggaaccttccctgggggtct	496

Query: 1023 gtgaaactgtgattgtgtatataaaaagcggcctccagcttggagaccagggcgggtaca 1082
|||||
Sbjct: 497 gtgaaactgtgattgtgtatataaaaagcggcctccagcttggagaccagggcgggtaca 556

Query: 1083 tactggagacagccaagagctgagtatataaaggagaggggaatgtgcaggaacagaggcg 1142
|||||
Sbjct: 557 tactggagacagccaagagctgagtatataaaggagaggggaatgtgcaggaacagaggcg 616

Query: 1143 tcttccctgggtctggcctcccgcttccctcactcttccctcttccctccacccccctagact 1202
|||||
Sbjct: 617 tcttccctgggtctggcctcccgcttccctcactcttccctcttccctccacccccctagact 676

Query: 1203 ct-gatcttacggatatcttgcctctgttccccatggagctccgaattcttgcgtgtgtg 1261
|||
Sbjct: 677 ctggatcttacggatatcttgcctctgttccccatggagctccgaattcttgcgtgtgtg 736

Query: 1262 tagatgagggggcgggggacggcgccaggcattgtccagacctggctcggggcccactgga 1321
|||||
Sbjct: 737 tagatgagggggcgggggacggcgccaggcattgtccagacctggctcggggcccactgga 796

Query: 1322 agcatccagaacagcaccaccatctagcggc 1352
|||||
Sbjct: 797 agcatccagaacagcaccaccatctagcggc 827

Score = 262 bits (136). Expect = 6e-67
Identities = 138/139 (99%)
Strand = Plus / Plus



Query: 481 ggacaggaggccccctccagaatggggaagggtatccctggcagagctctcccgagcaga 540
|||||
Sbjct: 3 ggacaggaggccccctccagaatggggaagggtatccctggcagagctctcccgagcaga 62

Query: 541 gtctccgatgccccgggaagcctgggagagaatgggagagatccccgaaaaggagagcagtcg 600
|||||
Sbjct: 63 gtctccgatgccccgggaagcctgggagagaatgggagagatccccgaaaaggagagcagtcg 122

Query: 601 tcacccaaaaaacagaagaa 619
|||||
Sbjct: 123 tcacccaaaaaacagaagaa 141

CFU time: 0.08 user secs. 0.10 sys. secs 0.18 total secs.

Gapped
Lambda K H
1.33 0.621 1.12

Gapped
Lambda K H
1.33 0.621 1.12

Matrix: blastn matrix:1 -2
Gap Penalties: Existence: 5, Extension: 2
Number of Hits to DB: 3
Number of Sequences: 0

Number of extensions: 3
Number of successful extensions: 3
Number of sequences better than 10.0: 1
length of query: 1717
length of database: 2,896,930,593
effective HSP length: 24
effective length of query: 1693
effective length of database: 2,896,930,569
effective search space: 4904503453317
effective search space used: 4904503453317
T: 0
A: 0
X1: 6 (11.5 bits)
X2: 10 (19.2 bits)
S1: 12 (23.8 bits)
S2: 20 (39.1 bits)

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96 91 0483.5

Human Genome Sciences, Inc.
Our Ref.: C2276 EP

February 7, 2002
JAE/AD/ONG

Pursuant to Rule 88 EPC, we herewith request correction of the obvious errors in Figure 1B. Please find enclosed new Figure 1B in which the following amendments have been carried out:

1. In Figure 1B currently on file, the last three amino acids encoded by nucleotides 1023 to 1031 have been accidentally omitted and are introduced in the amino acid sequence in new Figure 1B.

The amino acid sequence shown in Figure 1B corresponds to the open reading frame of the nucleic acid sequence. However, it is immediately evident that nucleotides 1023 to 1031 are also part of the open reading frame because a stop codon is not present in the sequence upstream of said nucleotides. Rather, the first stop codon in the nucleotide sequence is present downstream at nucleotides 1032 to 1034 (TGA).

Consequently, it is immediately evident that nothing else was intended than exhibiting the amino acid sequence of the entire open reading frame. Accordingly, the amino acid sequence of the entire open reading frame is offered as correction acid in new Figure 1B.

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2. In Figure 1B currently on file, the amino acids encoded by nucleotides 984 to 989 ("K" and "L") and by nucleotides 1020 to 1022 ("F") have been newly typed in new Figure 1B in order to improve the quality of said Figure. Thus, even from the amino acid sequence of Figure 1B currently on file, it is immediately evident that nothing else than what is offered as correction would have been intended.

In addition, the corrected amino acids encoded by the corresponding nucleic acid sequence under which they have been introduced.



Dr. Hans-Rainer Jaenichen
European Patent Attorney
Enclosure:

New Figure 1B

FIG. 1B

MATCH WITH FIG. 1A

721	GCCTACAGGCCCAAGGATATGCTGTCCGAATCCAGGATGCTGGAGTTTATCTGCTGTATA	780
	L Q A Q G Y G V R I Q D A G V Y L L Y S	
781	GCCAGGTCCTGTTTCAAGACGTGACTTTTACCATGGTCAAGTGGTGTCTCGAGAAGGCC	840
	Q V L F Q D V T F T M G Q V V S R E G Q	
841	AAGGAAGCCAGGAGACTCTATTCCGATGTATAGAAGTATGCCCTCCACCCGACCGGG	900
	G R Q E T L F R C I R S M P S H P D R A	
901	CCTACAACAGCTGCTATAGCGCAGGTGTCTTCCATTACACCAAGGGGATATTCTGAGTG	960
	Y N S C Y S A G V F H L H Q G D I L S V	
961	TCATAATTCCCCCGCCAGGGCGAAAGTTAACCTCTCTCCACATGGAACCTTCCTGGGGT	1020
	I I P P A R A K L N L S P H G T F L G F	
1021	TTGTGAAACTGTGATTGTGTTATAAAAGTGGCTCCAGCTTGGAAAGACCAGGGTGGGTA	1080
	V K L	
1081	CATACTGGAGACAGCCCAAGAGCTCAGTATATAAGGAGAGGGAATGTGCAGGAACAGAGG	1140
1141	CGTCTTCCTGGGTTTGGCTCCCCGTTCTCTCACTTTTCCCTTTTTCATTCCACCCCCCTAGA	1200
1201	CTTTGATTTTACGGATATCTTGTCTTCTTCCCCATGGAGCTCCGAATTCTTGCCTGTGT	1260
1261	GTAGATGAGCGCGCGGACCGCCCGCCAGCCATTGTCCACAGCTGGTGGGGCCCCACTGG	1320
1261	AAGCATCCAGAACAGCACCACTATACGGCGCGCTCTAGAGGATCCCTCGAGGGGCCCCA	1380
1381	AGCTTACGGGTGCA TGGACGTCATAGCTCTCTCCCTATAGTGAGTCGTATTATAAGCTA	1440
1441	GCTTGGGATCTTTTCTGAGGAACCTTACTTCTGTGGTGTGACATAATTGGACAAACTACC	1500
1501	TACAGAGATTTAAAGCTCTAAGGTAATAATAAAATTTTAAAGTGTATAATGTGTTAAACT	1560
1561	AGCTGCATATGCTTCTGCTTGCAGAGTTTGGCTTACTGAGTATGATTAATAAATATAT	1620
1621	ACACAGGAGCTAGTGATCTATGTTGCTTTTAGATCAAGCCCAAGGTTCATTCAGGCCCTCACC	1680
1681	TCAAGCTGTGATCATCATATCAGCATACAAATTGTGAG	1717

2 / 10

FIG. 1B

MATCH WITH FIG. 1A

721	GCCTACAGGCCCCAAGGATATGGTGTCCGAATCCAGGATGCTGGAGTTTATCTGCTGTATA	780
	L Q A Q G Y G V R I Q D A G V Y L L Y S	
781	GCCAGGTCCTGTTTCAAGACGTGACTTTTCAACCATGGTTCAGGTGGTGTCTCGAGAAGGCC	840
	Q V L F Q D V T F T M G Q V V S R E G Q	
841	AAGGAAGGCAGGAGACTCTATTCCGATGTATRAAGAAGTATGCCCTCCACCCCGACCCGG	900
	G R Q E T L F R C I R S M P S H P D R A	
901	CCTACAACAGCTCCTATAGCGCAGGTGTCTTCCATTACACCAAGGGGATATTTCTGAGTG	960
	Y N S C Y S A G V F H L H Q G D I L S V	
961	TCATAATTCCCGCGGCAAGGGCGAACTTAACCTCTCTCCACATGGAACCTTCTCTGGGGT	1020
	I I P P A R A ^K _L N L S P H G T F L G P	
1021	TTGTGAAACTGTGATTGTTTATAAAAGTGGCTCCAGCTTGGAGACCCAGGTTGGGTA	1080
	^V _K _L CATACTGGAGACACCCCAAGAGCTGAGTATATNAAGCAGAGGGAATGTGCAGGAACAGAGG	1140
1081	CGTCTTCTGGGTTTGGCTCCCGTTCTCTCACTTTTCCCTTTTTCATTCCCAACCCCTAGA	1200
1141	CTTTGATTTTACGCATATCTTGCTTCTGTCTCCCAATGGAGCTCCGAATCTTGGCGTGTGT	1260
1201	GTAGATGAGGGCGGGGACGGCGCCAGCCATTGTCACAGACCTGGTCGGGGCCCACTGG	1320
1261	AAGCATCCAGAACAGCACCACTTACCGCGCCCTCTAGAGGATCCCTCGAGGGGCCCA	1380
1321	AGCTTACGGGTGATCGGACCTCATAGCTCTCTCCCTATAGTGAGTCGTATTATAAGCTA	1440
1381	GCTTGGGATCTTTGTGAAGGAACCTTACTTCTGTGTGTGACATAATTGGACAAACTACC	1500
1441	TACAGAGATTTAAAGCTCTAAGCTAAATATAAAATTTTAAAGTGATATAATGTGTTAACT	1560
1501	AGCTGCATATGCTTCTGCTTTCAGAGTTTGGCTTACTGAGTATGATTTATGAAAATATTAT	1620
1561	ACACAGGAGCTAGTGATCTATGTTGCTTTTAGATCAAGCCAAAGGTCATTCAGGCCCTCAGC	1680
1621	TCAAGCTGTCTATCATATATCAGCATACAAATTGTGAG	1717



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Datum/Date

16-01-2002

Zeichen/Ref./Réf.

C 2276 EP

Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°.

96910483.5-2110/US9603774

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire

HUMAN GENOME SCIENCES, INC.

EXTENSION OF TIME LIMIT PURSUANT TO RULE 84 EPC

Examination procedure

With reference to your request, the time limit for replying to the communication dated 07.08.01 has been extended

by 2 months

to a total of 6 months,

from the date of notification of the above-mentioned communication.

Please note: To the extent that your request exceeded the above extension, your request has been refused.

Note:

The granting of extensions to time limits is governed by the implementing Regulations to the EPC and the Guidelines for Examination in the EPO, part E-VIII, 1.6.

If no reply to the communication is received in due time, the European patent application will be deemed to be withdrawn (Article 96(3) EPC).

For the Examining Division:

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17. Dez. 2001

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EP 96 91 0483.5-2110
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

December 13, 2001
Jae/ivj

Reference is made to the Communication dated August 7, 2001:

It is respectfully requested that the four-month period specified therein for filing
a reply be extended by two months, i.e. until

February 17, 2002.


Dr. Hans-Rainer Jaenichen
European Patent Attorney



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Application No. 96 910 483.5-2110	Ref. C 2276 EP	Date 07.08.2001
Applicant HUMAN GENOME SCIENCES, INC.		

Communication pursuant to Article 96(2) EPC

The examination of the above-identified application has revealed that it does not meet the requirements of the European Patent Convention for the reasons enclosed herewith. If the deficiencies indicated are not rectified the application may be refused pursuant to Article 97(1) EPC.

You are invited to file your observations and insofar as the deficiencies are such as to be rectifiable, to correct the indicated deficiencies within a period

of 4 months

from the notification of this communication, this period being computed in accordance with Rules 78(2) and 83(2) and (4) EPC.

Amendments to the description, claims and drawings are to be filed where appropriate within the said period in **three copies** on separate sheets (Rule 36(1) EPC).

Failure to comply with this invitation in due time will result in the application being deemed to be withdrawn (Article 96(3) EPC).



DEFFNER C A E
Primary Examiner
for the Examining Division

Enclosure(s): 2 page/s reasons (Form 2906)



The examination is being carried out on the **following application documents**:

Text for the Contracting States:

AT BE CH LI DE DK ES FI FR GB GR IE IT LU MC NL PT SE

Description, pages:

1-64 as published

Claims, No.:

1-21 as received on 19.10.1998 with letter of 19.10.1998

Drawings, sheets:

1/10-10/19 as published

- 1). Amended set of claims received on 19/10/98 introduce subject- matter which extends the content of the application as filed. Concerned are the polynucleotides encoding peptides of TNF delta and gamma as defined in claim 1 f) of the amended set of claims. No basis is apparent for the introduction of these polynucleotides which are not disclosed in the application as filed. Additionally, no basis is apparent for the fragment of at least 30 bases in length, see claim 1 point g). Therefore requirements of Article 123(2) are contravened.
- 2). Claim 1 point f) does not meet the requirements of Article 56 EPC because it also relates to inactive variants which would not solve the underlying problem of the present application defined with the provision of a further members of the TNF family.
- 3). To avoid cross- reactivity with other proteins sharing eventually same epitopes with present protein claimed present claim 11 should be directed to specific antibodies.



- 4). Claims 13, 17 and 21 relate to antagonists, their use and method for the production of a pharmaceutical composition containing it. The claims relate to subject matter defined in the result to be achieved i.e. in functional features. However, it is not clear from the application which structural feature such an inhibitor requires. Maybe even known compounds would fall under the scope of the claims. Therefore the requirements of Article 84 EPC are not met because the matter for which protection is sought is not sufficiently defined.
- 5). The last phrase on page 64 and on page 8, lines 3 to 6, render the scope of the claims unclear when used to interpret it (Article 84 EPC).
- 6). It is not clear which is the TNF activity as mentioned in claim 1h) (Article 84 EPC).
- 7.). Remaining subject- matter not concerned by above comments is considered to be novel. However, according to applicant's statement on page 4, first paragraph, the novel TNF variants have been identified by homology to already known TNF sequences. With respect to this it appears that any skilled person could have identified present TNF variants and, therefore, the present subject- matter appears not to involve an inventive step (Articles 54 and 56 EPC).
- 8). For purpose of information it is noted that an objection of lack of unity of invention is not raised because of the relative high degree of homology between both factors (97% in 729 bp overlap).
- 9). When filing amended claims the applicant should at the same time bring the description into conformity with the amended claims. Care should be taken during revision, especially of the introductory portion and any statements of problem or advantage, not to add subject-matter which extends beyond the content of the application as originally filed (Article 123(2) EPC).

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Our Ref.: C2276 EP

March 31, 1999
C/KT/99/90142

Referring to the Communication pursuant to Art. 96 (1) and
Rule 51 (1) EPC applicants declare their consent to proceed
further with the application.


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Bob Verboom 15. 4. 1999



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96910483.5-1270- PCT/US9603774

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire

HUMAN GENOME SCIENCES, INC.

PROCEEDING FURTHER WITH THE EUROPEAN PATENT APPLICATION PURSUANT TO
ARTICLE 96(1) AND RULE 51(1) EPC

A supplementary European search report has been drawn up concerning
the above European patent application (publication no. 0897390).

Since you have filed a request for examination prior to the trans-
mission of the supplementary European search report, you are hereby
invited to indicate within

TWO MONTHS

of notification of this invitation whether you desire to proceed
further with the European patent application.

If you do not indicate in due time that you desire to proceed further
with the European patent application, it will be deemed to be withdrawn
(Art. 96(3) EPC).

If you wish you may comment on the supplementary European search report
and amend, where appropriate, the description, claims and drawings
(Rule 51(1) EPC).

RECEIVING SECTION



REGISTERED LETTER

EPO Form 1224 04.85		7001007 15/02/99	
96910483.5	DMEX	MO2	006



P B 5818 - Patentaan 2
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Europäisches
Patentamt

Zweigstelle
in Den Haag
Recherchen-
abteilung

European
Patent Office

Branch at
The Hague
Search
division

Office européen
des brevets

Département à
La Haye
Division de la
recherche

12

VOSSIUS & PARTNER
Postfach 86 07 67
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ALLEMAGNE

COPY

Datum/Date

19. 02. 1999

Zeichen/Ref./Réf.

C 2276 EP

Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°.

96910483.5

Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire

HUMAN GENOME SCIENCES, INC.

COMMUNICATION

The European Patent Office herewith transmits

- ☐ the European search report
- ☐ the declaration under Rule 45 EPC
- ☐ the partial European search report under Rule 45 EPC
- ☒ the supplementary European search report concerning the international application under Article 157(2) EPC relating to the above-mentioned European patent application. ~~Copies of the documents cited in the search report are enclosed.~~

The following specifications given by the applicant have been approved by the Search Division :

- ☐ Abstract
- ☐ Title
- ☐ Figure
- ☐ The abstract was modified by the Search Division and the definitive text is attached to this communication.
- ☐ The following figure will be published with the abstract, since the Search Division considers that it better characterises the invention than the one indicated by the applicant.

Figure:

- ☐ Additional copy(copies) of the documents cited in the European search report.

REFUND OF THE SEARCH FEE

If applicable under Article 10 Rules relating to fees, a separate communication from the Receiving Section on the refund of the search fee will be sent later.



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EPO Form 1507 02.93



European Patent
Office

**SUPPLEMENTARY
PARTIAL EUROPEAN SEARCH REPORT**

Application Number

which under Rule 45 of the European Patent Convention shall be considered, for the purposes of subsequent proceedings, as the European search report

EP 96 91 0483

DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
	No further relevant documents disclosed -----		C07H21/02 C07H21/04 C07K1/00 C07K14/00 C12N5/00 C12N15/09 C12N15/63 C07K14/525
			TECHNICAL FIELDS SEARCHED (Int.Cl.6)
			C07K
INCOMPLETE SEARCH			
<p>The Search Division considers that the present application, or some or all of its claims, does/do not comply with the EPC to such an extent that a meaningful search into the state of the art cannot be carried out, or can only be carried out partially, for the following claims:</p> <p>Claims searched completely :</p> <p>Claims searched incompletely :</p> <p>Claims not searched :</p> <p>Reason for the limitation of the search:</p> <p>see sheet C</p>			
Place of search		Date of completion of the search	Examiner
MUNICH		14 January 1999	Deffner, C-A
CATEGORY OF CITED DOCUMENTS			
<p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons</p> <p>& : member of the same patent family, corresponding document</p>			



European Patent
Office

INCOMPLETE SEARCH
SHEET C

Application Number

EP 96 91 0483

Claim(s) searched completely:
1-12,14-16,18,19,21

Claim(s) not searched:
13,17,20

Reason for the limitation of the search:

Claims 13, 17 and 21 relate to antagonists, their use and method for the production of a pharmaceutical composition containing it. The claims relate to subject matter defined in the result to be achieved i.e. in functional features. However, it is not clear from the application which structural feature such an inhibitor requires. Maybe even known compounds would fall under the scope of the claims. Therefore a meaningful search is not possible.



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Datum/Date

13/01/99

Zeichen/Ref./Réf.	Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°
C 2276 EP PCT/US9603774	96910483.5-1270 / 0897390
Anmelder/Applicant/Demandeur/Patentinhaber/Proprietor/Titulaire	
HUMAN GENOME SCIENCES, INC.	

**NOTIFICATION OF EUROPEAN PUBLICATION NUMBER AND INFORMATION
ON THE APPLICATION OF ARTICLE 67(3) EPC**

The provisional protection under Article 67(1) and (2) EPC in the individual Contracting States becomes effective only when the conditions referred to in Article 67(3) EPC have been fulfilled (for further details, see information brochure of the European Patent Office "National Law relating to the EPC" and additional information in the Official Journal of the European Patent Office).

Pursuant to Article 158(1) EPC the publication under Article 21 PCT of an international application for which the European Patent Office is a designated Office takes the place of the publication of a European patent application.

The bibliographic data of the above-mentioned Euro-PCT application will be published on 24.02.99 in Section I.1 of the European Patent Bulletin.

The European publication number is 0897390.

In all future communications to the European Patent Office, please quote the application number plus Directorate number.

RECEIVING SECTION



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Patentanwälte

Vossius & Partner POB 86 07 67 81834 München Germany

To the

European Patent Office

Munich

PATENTANWÄLTE
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 (bis 1992; danach in anderer Kanzlei)
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 (Marken - Trademarks)

006
 EP Patent Application No. 96 91 0483.5
 Human Genome Sciences, Inc.
 Our Ref.: C 2276 EP

October 19, 1998
 Jae/PST/es

Further to our petition dated October 13, 1998, we enclose herewith a new set of claims 1 to 21 which should replace claims 1 to 21 filed on October 13, 1998. The new claims set is identical to that of October 13, 1998, except for claim 1, section (f) wherein reference to SEQ ID NO:2 and SEQ ID NO:4, respectively, has been replaced with the reference to the corresponding amino acid sequences of Figure 1 and 2, respectively.

It is submitted that no new subject matter has been added to the application as original filed.

Yours faithfully,

Dr. Hans-Rainer Jaenichen
 European Patent Attorney

S. Hunold 09-11-1998

Enc.

New claims 1 to 21, in triplicate

EP 96 91 0483.5
PCT/US96/03774
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

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VOSSIUS & PARTNER
PATENTANWÄLTE
SIEBERTSTR. 4
81675 MÜNCHEN

19. Okt. 1998

CLAIMS

1. A polynucleotide selected from the group consisting of
 - (a) polynucleotides encoding at least the mature form of the polypeptide having the deduced amino acid sequence as shown in Figure 1 or 2;
 - (b) polynucleotides having the coding sequence as shown in Figure 1 or 2 encoding at least the mature form of the polypeptide;
 - (c) polynucleotides encoding the polypeptide having the amino acid sequence of at least the mature form of the polypeptide encoded by the cDNA contained in ATCC 97377 or ATCC 97457;
 - (d) polynucleotides having the coding sequence of the cDNA contained in ATCC 97377 or ATCC 97457 encoding at least the mature form of the polypeptide;
 - (e) polynucleotides encoding an amino acid sequence encoded by a polynucleotide of any one of (a) to (d), in which 1 to 5 or 5 to 10 amino acids are substituted, deleted or added, in any combinations;
 - (f) polynucleotides encoding amino acids 72-96, amino acids 115-126, amino acids 130-167, and/or amino acids 173-182 of the amino acid sequence shown in Figure 1, and/or amino acids 52-61, amino acids 65-102, amino acids 108-117, and/or amino acids 132-138 of the amino acid sequence shown in Figure 2;
 - (g) polynucleotides comprising a fragment of at least 30 bases in length of a polynucleotide of any one of (a) to (d); and
 - (h) polynucleotides which are at least 70% identical to a polynucleotide as defined in any one of (a) to (d) or (g) and which encode a polypeptide having TNF delta or TNF epsilon activity;
 - (i) polynucleotides encoding a polypeptide which is at least 70% identical to a polypeptide encoded by a polynucleotide of any one of (a) to (d);or the complementary strand of such a polynucleotide.
2. The polynucleotide of claim 1 which is DNA or RNA.

M 20 10 99

3. The DNA of claim 2 which is genomic DNA.
4. The polynucleotide of any one of claims 1 to 3 which is fused to a heterologous polynucleotide.
5. A vector containing the polynucleotide of any one of claims 1 to 4.
6. The vector of claim 5 in which the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
7. A host cell genetically engineered with the polynucleotide of any one of claims 1 to 4 or the vector of claim 5 or 6.
8. A process for producing a polypeptide having TNF delta or TNF epsilon activity comprising: culturing the host cell of claim 7 and recovering the polypeptide encoded by said polynucleotide from the culture.
9. A process for producing cells capable of expressing a polypeptide having TNF delta or TNF epsilon activity comprising genetically engineering cells with the vector of claim 5 or 6.
10. A polypeptide having the amino acid sequence encoded by a polynucleotide of any one of claims 1 to 4 or obtainable by the process of claim 8.
11. An antibody against the polypeptide of claim 10.
12. A nucleic acid molecule which specifically hybridizes to a polynucleotide of any one of claims 1 to 4.
13. An antagonist/inhibitor of the polypeptide of claim 10.
14. A pharmaceutical composition comprising the polynucleotide of any one of claims 1 to 4, the polypeptide of claim 10 or a DNA encoding and capable of



expressing said polypeptide in vivo or the antagonist/inhibitor of claim 13 and optionally a pharmaceutically acceptable carrier.

15. A diagnostic composition comprising the polynucleotide of any one of claims 1 to 4, the nucleic acid molecule of claim 12 or the antibody of claim 11.
16. Use of the polypeptide of claim 10 or the polynucleotide of any one of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment of neoplasia, for wound-healing, for the treatment of restenosis, for regulating hematopoiesis in endothelial cell development, for stimulating an immune response against parasitic, bacterial or viral infections, or for the treatment and/or prevention of autoimmune diseases.
17. Use of the antagonist/inhibitor of claim 13 for the preparation of a pharmaceutical composition for the treatment of cachexia, cerebral malaria, rheumatoid arthritis, for the prevention of graft-host rejection, for inhibiting bone resorption, for the treatment and/or prevention of osteoporosis, or for the treatment of endotoxic shock.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 10 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 10 in a sample derived from a host.
20. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 10 comprising:
 - (a) contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF delta polypeptide and a compound under conditions to permit binding to the receptor; and

N 20 10 99

- (b) determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.
21. A method for the production of a pharmaceutical composition comprising the steps of the method of claim 20 and (c) formulating the compound identified in step (b) in a pharmaceutically acceptable form.

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VOSSIUS & PARTNER

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EP Patent Application No. 96 91 0483.5
Human Genome Sciences, Inc.
Our Ref.: C 2276 EP

October 13, 1998
Jae/PST/es

In addition to the inventors listed on the cover sheet of WO 97/33902

DILLON, Patrick J.
1055 Snipe Court
Carlsbad, California 92009
United States of America

JNVT 04

S. Hunold 09-11-1998

is designated as an inventor for the above-mentioned application.

Munich, October 13, 1998


Dr. Hans-Rainer Jaenichen



An das Europäische Patentamt

To the European Patent Office

A l'Office européen des brevets

15

**Eintritt in die regionale
Phase vor dem EPA
als Bestimmungsamt
oder ausgewähltem Amt**

**Entry into the regional
phase before the EPO
as designated or elected
Office**

**Entrée dans la phase
régionale devant l'OEB
agissant en qualité d'Office
désigné ou élu**

Europäische Anmeldenummer oder, falls nicht bekannt, PCT-Aktenzeichen oder PCT-Veröffentlichungsnummer 006	European application number, or, if not known, PCT application or publication number EP 96 91 0483.5 (PCT/US96/03774)	Número de depósito de la demanda de brevet europeo ou, à défaut numéro de dépôt PCT ou de publication PCT
Zeichen des Anmelders oder Vertreters (max. 15 Positionen)	Applicant's or representative's reference (max 15 spaces) C 2276 EP	Référence du demandeur ou du mandataire (15 caractères ou espaces au maximum)
<input checked="" type="checkbox"/> 1. Anmelder Die Angaben über den (die) Anmelder sind in der internationalen Veröffentlichung enthalten oder vom Internationalen Büro nach der internationalen Veröffentlichung vermerkt werden. <input type="checkbox"/> Änderungen, die das Internationale Büro noch nicht vermerkt hat, sind auf einem Zusatzblatt angegeben. Zustellanschrift (siehe Merkblatt II, 1)	1. Applicant Indications concerning the applicant(s) are contained in the international publication or recorded by the International Bureau after the international publication. Changes which have not yet been recorded by the International Bureau are set out on an additional sheet. Address for correspondence (see Notes II, 1)	1. Demandeur Les indications concernant le(s) demandeur(s) figurent dans la publication internationale ou ont été enregistrées par le Bureau international après la publication internationale. Les changements qui n'ont pas encore été enregistrés par le Bureau international sont indiqués sur une feuille additionnelle. Adresse pour la correspondance (voir notice II, 1)
2. Vertreter Name (Nur einen Vertreter angeben, der in das europäische Patentregister eingetragen und an den zugestellt wird) Geschäftsanschrift Telefon Telefax Telex <input type="checkbox"/> Weitere(r) Vertreter auf Zusatzblatt	2. Representative Name (Name only one representative who is to be listed in the Register of European Patents and to whom notification is to be made) VOSSIUS & PARTNER (Nr. 31) Address of place of business Postfach 86 07 67 81634 Munich DE Telephone +49-89-4 13 04-0 Fax Telex +49-89-4 13 04-111 Additional representative(s) on additional sheet S. Hunold	2. Mandataire Nom (N'indiquer qu'un seul mandataire, qui sera inscrit au Registre européen des brevets et auquel signification sera faite) Adresse professionnelle Téléphone Téléfax Telex 09 -11- 1998 Autre(s) mandataire(s) sur feuille additionnelle 100311.0
3. Vollmacht <input type="checkbox"/> Einzelvollmacht ist beigelegt <input type="checkbox"/> Allgemeine Vollmacht ist registriert unter Nummer: <input type="checkbox"/> Allgemeine Vollmacht ist eingereicht, aber noch nicht registriert. <input type="checkbox"/> Die beim ERA als PCT-Anmeldeamt eingereichte Vollmacht schließt ausdrücklich die regionale Phase ein.	3. Authorisation Individual authorisation is attached. General authorisation has been registered under No: A general authorisation has been filed, but not yet registered. The authorisation filed with the EPO as PCT receiving Office expressly includes the regional phase.	3. Pouvoir Un pouvoir spécial est joint Un pouvoir général a été enregistré sous le n° Un pouvoir général a été déposé mais n'est pas encore enregistré. Le pouvoir général tel que déposé à l'OEB agissant en qualité d'office récepteur au titre du PCT s'applique expressément à la phase régionale.

<p><input checked="" type="checkbox"/> 4. Prüfungsantrag Hiermit wird die Prüfung der Anmeldung gemäß Art. 94 EPU beantragt. Die Prüfungsgebühr wird (wurde) entrichtet.</p> <p><i>Prüfungsantrag in einer zugelassenen Nichtamtssprache (siehe Merkblatt III, 6.2):</i></p> <p>_____</p>	<p>4. Request for examination Examination of the application under Art. 94 EPC is hereby requested. The examination fee is being (has been, will be) paid</p> <p><i>Request for examination in an admissible non-EPO language (see Notes III, 6.2):</i></p> <p>_____</p>	<p>4. Requête en examen Il est demandé que la demande de brevet soit examinée, conformément à l'art. 94 CBE. Il est (a été, sera) procédé au paiement de la taxe d'examen.</p> <p><i>Requête en examen dans une langue non officielle autorisée (voir notice III, 6.2):</i></p> <p>_____</p>
<p><input checked="" type="checkbox"/> 5. Abschriften Zusätzliche Abschrift(en) der im ergänzenden europäischen Recherchenbericht angeführten Schriftstücke wird (werden) beantragt.</p> <p>Anzahl der zusätzlichen Sätze von Abschriften</p> <p>_____</p>	<p>5. Copies Additional copy (copies) of the documents cited in the supplementary European search report is (are) requested.</p> <p>Number of additional sets of copies</p> <p>1</p>	<p>5. Copies Prère de fournir une (des) copie(s) supplémentaire(s) des documents cités dans le rapport complémentaire de recherche européenne.</p> <p>Nombre de jeux supplémentaires de copies</p> <p>_____</p>
<p>6. Für das Verfahren vor dem EPA bestimmte Unterlagen</p> <p>6.1 Dem Verfahren vor dem EPA als Bestimmungsamt (PCT I) sind folgende Unterlagen zugrunde zu legen.</p> <p><input checked="" type="checkbox"/> die vom Internationalen Büro veröffentlichten Anmeldungsunterlagen (mit allen Ansprüchen, Beschreibung und Zeichnungen), gegebenenfalls mit den geänderten Ansprüchen nach Art. 19 PCT</p> <p><input type="checkbox"/> soweit sie nicht ersetzt werden durch die in drei Stücken beigefügten Änderungen.</p> <p><i>Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!</i></p> <p>6.2 Dem Verfahren vor dem EPA als ausgewähltem Amt (PCT II) sind folgende Unterlagen zugrunde zu legen:</p> <p><input checked="" type="checkbox"/> die dem Internationalen vorläufigen Prüfungsbericht zugrunde gelegten Unterlagen, einschließlich seiner eventuellen Anlagen (Solche Anlagen müssen immer in drei Stücken beigefügt werden)</p> <p><input checked="" type="checkbox"/> soweit sie nicht ersetzt werden durch die in drei Stücken beigefügten Änderungen.</p> <p><i>Falls nötig, sind Klarstellungen auf einem Zusatzblatt einzureichen!</i></p> <p><input checked="" type="checkbox"/> Sind dem EPA als mit der internationalen vorläufigen Prüfung beauftragten Behörde Versuchsberichte zugegangen, dürfen diese dem Verfahren vor dem EPA zugrunde gelegt werden.</p>	<p>6. Documents intended for proceedings before the EPO</p> <p>6.1 Proceedings before the EPO as designated Office (PCT I) are to be based on the following documents:</p> <p>the application documents published by the International Bureau (with all claims, description and drawings), where applicable with amended claims under Art. 19 PCT</p> <p>unless replaced by the amendments enclosed in triplicate.</p> <p><i>Where necessary, clarifications must be submitted on a separate sheet!</i></p> <p>6.2 Proceedings before the EPO as elected Office (PCT II) are to be based on the following documents:</p> <p>the documents on which the International preliminary examination report is based, including its possible annexes (Such annexes must always be filed in triplicate)</p> <p>unless replaced by the amendments enclosed in triplicate.</p> <p><i>Where necessary, clarifications must be submitted on a separate sheet!</i></p> <p>If the EPO as International Preliminary Examining Authority has received test reports, these may be used as the basis of proceedings before the EPO</p>	<p>6. Pièces destinées à la procédure devant l'OEB</p> <p>6.1 La procédure devant l'OEB agissant en qualité d'office désigné (PCT I) doit se fonder sur les pièces suivantes :</p> <p>les pièces de la demande publiée par le Bureau international (avec toutes les revendications, la description et les dessins), éventuellement avec les revendications modifiées conformément à l'article 19 du PCT</p> <p>dans la mesure où elles ne sont pas remplacées par les modifications jointes en trois exemplaires.</p> <p><i>Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!</i></p> <p>6.2 La procédure devant l'OEB agissant en qualité d'office élu (PCT II) doit se fonder sur les pièces suivantes :</p> <p>les pièces sur lesquelles se fonde le rapport d'examen préliminaire international, y compris ses annexes éventuelles (De telles annexes sont toujours à joindre en trois exemplaires)</p> <p>dans la mesure où elles ne sont pas remplacées par les modifications jointes en trois exemplaires.</p> <p><i>Le cas échéant, des explications doivent être jointes sur une feuille additionnelle!</i></p> <p>Si l'OEB, agissant en qualité d'administration chargée de l'examen préliminaire international, a reçu des rapports d'essais, ils peuvent constituer la base de la procédure devant l'OEB.</p>

7. Übersetzungen

Beigefügt sind die nachfolgend angekreuzten Übersetzungen in einer der Amtssprachen des EPA (Deutsch, Englisch, Französisch).

- Im Verfahren vor dem EPA als **Bestimmungsamt oder ausgewähltem Amt (PCT I + II)**

☐

Übersetzung der **ursprünglich eingereichten internationalen Anmeldung** (Beschreibung, Ansprüche, etwaige Textbestandteile in den Zeichnungen), der veröffentlichten Zusammenfassung, und etwaiger Angaben über Mikroorganismen nach Regel 13^{ter}.3 und 13^{ter}.4 PCT, **in drei Stücken**

☐

Übersetzung der **prioritätsbegründenden Anmeldung(en)**, **in einem Stück**

- **Zusätzlich** im Verfahren vor dem EPA als **Bestimmungsamt (PCT I)**:

☐

Übersetzung der nach Art. 19 PCT **geänderten Ansprüche** nebst Erklärung, falls diese dem Verfahren vor dem EPA zugrunde gelegt werden sollen (siehe Feld 6), **in drei Stücken**

- **Zusätzlich** im Verfahren vor dem EPA als **ausgewähltem Amt (PCT II)**.

☐

Übersetzung der **Anlagen zum internationalen vorläufigen Prüfungsbericht**, **in drei Stücken**

7. Translations

Translations in one of the official languages of the EPO (English, French, German) are enclosed as crossed below.

- In proceedings before the EPO as **designated or elected Office (PCT I + II)**.

Translation of the **international application** (description, claims, any text in the drawings) **as originally filed**, of the abstract as published and of any indication under Rule 13^{ter}.3 and 13^{ter}.4 PCT regarding micro-organisms, **in triplicate**

Translation of the **priority application(s)**, **in one copy**

- **In addition**, in proceedings before the EPO as **designated Office (PCT I)**

Translation of **amended claims** and any statement under Art. 19 PCT, if the claims as amended are to form the basis for the proceedings before the EPO (see Section 6), **in triplicate**

- **In addition**, in proceedings before the EPO as **elected Office (PCT II)**:

Translation of any **annexes to the international preliminary examination report**, **in triplicate**

7. Traductions

Vous trouverez ci-jointes les traductions cochées ci-après dans l'une des langues officielles de l'OEB (allemand, anglais, français).

- Dans la procédure devant l'OEB agissant en qualité d'**Office désigné ou élu (PCT I + II)**

Traduction de la **demande internationale telle que déposée initialement** (description, revendications, textes figurant éventuellement dans les dessins), de l'abrégé publié, et de toutes indications visées aux règles 13^{ter}.3 et 13^{ter}.4 du PCT concernant les micro-organismes, **en trois exemplaires**

Traduction de la (des) **demande(s) ouvrant le droit de priorité**, **en un exemplaire**

- **De plus**, dans la procédure devant l'OEB agissant en qualité d'**office désigné (PCT I)**

Traduction des **revendications modifiées** et de la déclaration faite conformément à l'article 19 du PCT, si la procédure devant l'OEB doit être fondée sur les revendications modifiées (cf. rubrique 6), **en trois exemplaires**

- **De plus**, dans la procédure devant l'OEB agissant en qualité d'**office élu (PCT II)**:

Traduction des **annexes du rapport d'examen préliminaire international**, **en trois exemplaires**

8. Biologisches Material

Die Erfindung betrifft biologisches Material oder seine Verwendung, das nach Regel 28 EPÜ hinterlegt worden ist

☒
☒

Die **Angaben nach Regel 28(1)(c) EPÜ** sind in der internationalen Veröffentlichung oder in der gemäß Feld 7 eingereichten Übersetzung enthalten auf.

Seite(n) / Zeile(n)

8. Biological material

The invention relates to and/or uses biological material deposited under Rule 28 EPC.

The **particulars referred to in Rule 28(1)(c) EPC** are given in the international publication or in the translation submitted under Section 7 on

page(s) / line(s)

8. Matière biologique

L'invention concerne et/ou utilise la matière biologique, déposée conformément à la règle 28 CBE

Les **indications visées à la règle 28(1)(c) CBE** figurent dans la publication internationale ou dans une traduction produite conformément à la rubrique 7 à la / aux.

page(s) / ligne(s)

Page 23, lines 18-21

Die **Empfangsbescheinigung(en)** der Hinterlegungsstelle

☒

ist (sind) beigefügt

☐

wird (werden) nachgereicht

☐

Verzicht auf die Verpflichtung des Antragstellers nach Regel 28(3) auf gesondertem Schriftstück

The **receipt(s) of deposit** issued by the depositary institution

is (are) enclosed

will be filed at a later date

Waiver of the right to an undertaking from the requester pursuant to Rule 28(3) attached

Le(s) **récépissé(s) de dépôt** délivré(s) par l'autorité de dépôt

est (sont) joint(s)

sera (seront) produit(s) ultérieurement

Renonciation, sur document distinct, à l'engagement du requérant au titre de la règle 28(3).

9. Nucleotid- und Aminosäuresequenzen

☐ Die nach Regeln 5.2 und 13^{ter} PCT sowie Regel 104b (3a) EPU erforderlichen Unterlagen liegen dem EPA bereits vor.

☒ Das schriftliche Sequenzprotokoll wird anlegend in einer Amtssprache des EPA nachgereicht.

☒ Das Sequenzprotokoll geht nicht über den Inhalt der Anmeldung in der ursprünglich eingereichten Fassung hinaus

☒ Der vorgeschriebene maschinenlesbare Datenträger ist beigelegt.

☒ Die auf dem Datenträger gespeicherte Information stimmt mit dem schriftlichen Sequenzprotokoll überein.

9. Nucleotide and amino acid sequences

The items necessary in accordance with Rules 5.2 and 13^{ter} PCT and Rule 104b (3a) EPC have already been furnished to the EPO.

The written sequence listing is furnished herewith in an official language of the EPO.

The sequence listing does not include matter which goes beyond the content of the application as filed

The prescribed machine-readable data carrier is enclosed.

The information recorded on the data carrier is identical to the written sequence listing.

9. Séquences de nucléotides et d'acides aminés

Les pièces requises selon les règles 5.2 et 13^{ter} PCT et la règle 104^{ter} (3^{ème}) CBE ont déjà été déposées auprès de l'OEB

La liste de séquences écrite est produite ci-joint dans une des langues officielles de l'OEB

La liste de séquences ne contient pas d'éléments s'étendant au-delà du contenu de la demande telle qu'elle a été déposée

Le support de données prescrit, déchiffable par machine, est annexé

L'information figurant sur le support de données est identique à celle que contient la liste de séquences écrite.

10. Benennungsgebühren

10.1 Benennungsgebühren werden für nachstehende in der internationalen Anmeldung bestimmte Vertragsstaaten des EPU entrichtet:

<input checked="" type="checkbox"/>	AT	Österreich
<input checked="" type="checkbox"/>	BE	Belgien
<input checked="" type="checkbox"/>	CH/LI	Schweiz und Liechtenstein
<input checked="" type="checkbox"/>	DE	Deutschland
<input checked="" type="checkbox"/>	DK	Dänemark
<input checked="" type="checkbox"/>	ES	Spanien
<input checked="" type="checkbox"/>	FI	Finnland
<input checked="" type="checkbox"/>	FR	Frankreich
<input checked="" type="checkbox"/>	GB	Vereinigtes Königreich
<input checked="" type="checkbox"/>	GR	Griechenland
<input checked="" type="checkbox"/>	IE	Irland
<input checked="" type="checkbox"/>	IT	Italien
<input checked="" type="checkbox"/>	LU	Luxemburg
<input checked="" type="checkbox"/>	MC	Monaco
<input checked="" type="checkbox"/>	NL	Niederlande
<input checked="" type="checkbox"/>	PT	Portugal
<input checked="" type="checkbox"/>	SE	Schweden

☐ _____ 1)

☐ _____ 1)

10. Designation fees

10.1 Designation fees are paid in respect of the following EPC Contracting States designated in the international application for a European patent

Austria
Belgium
Switzerland and Liechtenstein
Germany
Denmark
Spain
Finland
France
United Kingdom
Greece
Ireland
Italy
Luxembourg
Monaco
Netherlands
Portugal
Sweden

_____ 1)

_____ 1)

10. Taxes de désignation

10.1 Les taxes de désignation sont acquittées pour ceux des Etats contractants de la CBE désignés dans la demande internationale qui sont indiqués ci-après:

Autriche
Belgique
Suisse et Liechtenstein
Allemagne
Danemark
Espagne
Finlande
France
Royaume-Uni
Grèce
Irlande
Italie
Luxembourg
Monaco
Pays-Bas
Portugal
Suède

_____ 1)

_____ 1)

☒ 10.2 Derzeit ist nicht beabsichtigt, Benennungsgebühren für die in Feld 10.1 nicht angekreuzten, aber in der internationalen Anmeldung bestimmten Vertragsstaaten des EPU zu entrichten. Insoweit wird auf die Zustellung einer Mitteilung nach Regel 85a(1) EPU verzichtet. Sofern diese Benennungsgebühren nicht bis zum Ablauf der in Regel 85a(2) EPU vorgesehenen Nachfrist entrichtet werden, wird beantrag, von einer Mitteilung nach Regel 69(1) EPU abzusehen.

10.2 At present it is not intended to pay designation fees for the EPC Contracting States not marked with a cross under 10.1 but designated in the international application. No communication under Rule 85a(1) EPC in respect of these designation fees need be notified. If they have not been paid by the time the period of grace allowed in Rule 85a(2) EPC expires, it is requested that no communication be sent under Rule 69(1) EPC.

10.2 Il n'est pas actuellement envisagé d'acquitter les taxes de désignation pour les Etats contractants de la CBE qui ne sont pas cochés sous la rubrique 10.1, mais qui sont désignés dans la demande internationale. Le demandeur renonce ainsi à la notification prévue à la règle 85bis(1) CBE. Si ces taxes de désignation ne sont pas acquittées à l'expiration du délai supplémentaire prévu à la règle 85bis(2) CBE, il est demandé de s'abstenir d'envoyer une notification, établie conformément à la règle 69(1) CBE

1) Vorgesehen für die Eintragung weiterer Vertragsstaaten des EPU, für die der PCT oder das EPU nach Drucklegung dieses Formblatts in Kraft tritt, und die in der internationalen Anmeldung für ein europäisches Patent bestimmt waren

1) Space for any other EPC Contracting States which may become PCT or EPC Contracting States after this form has been printed and which were designated for a European patent in the international application

1) Prévu pour l'inscription d'autres Etats contractants de la CBE à l'égard desquels le PCT ou la CBE entrera en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale pour un brevet européen

- ☒ **11. Erstreckung des europäischen Patents**
Diese Anmeldung gilt auch als Erstreckungsantrag hinsichtlich aller in der internationalen Anmeldung bestimmten Nicht-Vertragsstaaten des EPÜ, mit denen bei Einreichung der internationalen Anmeldung »Erstreckungsabkommen« in Kraft waren.
Die Erstreckung wird jedoch nur wirksam, wenn die vorgeschriebene Erstreckungsgebühr entrichtet wird. Der Anmelder beabsichtigt, die Erstreckungsgebühr für die nachfolgend angekreuzten Staaten zu entrichten.

- ☐ SI Slowenien (* ab 1. März 1994)
☐ LT Litauen (* ab 5. Juli 1994)
☐ LV Lettland (* ab 1. Mai 1995)
☐ AL Albanien (* ab 1. Februar 1996)
☐ RO Rumänien (* ab 15. Oktober 1996)

1) Platz für Staaten, mit denen »Erstreckungsabkommen« nach Drucklegung dieses Formblatts in Kraft treten und die in der internationalen Anmeldung bestimmt waren

- 11. Extension of the European patent**
This application is also considered as being a request for extension to all the non-Contracting States to the EPC designated in the international application with which "extension agreements" were in force on the date of filing the international application.
However, the extension only takes effect if the prescribed extension fee is paid.
The applicant intends to pay the extension fee for the States marked with a cross below:

- Slovenia (* as of 1 March 1994)
Lithuania (* as of 5 July 1994)
Latvia (* as of 1 May 1995)
Albania (* as of 1 February 1996)
Romania (* as of 15 October 1996)

1) Space for States with which "extension agreements" enter into force after this form has been printed and which were designated in the international application

- 11. Extension des effets du brevet européen**
La présente demande est également réputée demande d'extension à tous les Etats non adhérents à la CBE désignés dans la demande internationale, avec lesquels existaient, lors du dépôt de la demande, des »accords d'extension« .
Toutefois l'extension ne produit ses effets que si la taxe d'extension prescrite est acquittée.
Le demandeur se propose actuellement d'acquitter la taxe d'extension pour les Etats dont le nom est coché ci-après

- Slovénie (* à compter du 1^{er} mars 1994)
Lituanie (* à compter du 5 juillet 1994)
Lettonie (* à compter du 1^{er} mai 1995)
Albanie (* à compter du 1^{er} février 1996)
Roumanie (* à compter du 15 octobre 1996)

1) Prévu pour des Etats à l'égard desquels des »accords d'extension« entreront en vigueur après l'impression du présent formulaire et qui ont été désignés dans la demande internationale

- 12. Automatischer Abbuchungsauftrag (Nur möglich für Inhaber von beim EPA geführten laufenden Konten)**

- ☐ Das EPA wird beauftrag, nach Maßgabe der Vorschriften über das automatische Abbuchungsverfahren fällige Gebühren und Auslagen vom untenstehenden laufenden Konto abzubuchen

Nummer des laufenden Kontos /
Name des Kontoinhabers

- 12. Automatic debit order (for EPO deposit account holders only)**

The EPO is hereby authorised, under the Arrangements for the automatic debiting procedure, to debit from the deposit account below any fees and costs falling due.

Deposit account number / Account holder's name

- 12. Ordre de prélèvement automatique (uniquement possible pour les titulaires de comptes courants ouverts auprès de l'OEB)**

Par la présente, il est demandé à l'OEB de prélever du compte courant ci-dessous les taxes et frais venant à échéance, conformément à la réglementation relative au prélèvement automatique

N° du compte courant / Nom du titulaire du compte

- ☒ **13 Eventuelle Rückzahlungen auf das beim EPA geführte laufende Konto Nummer**

Name des Kontoinhabers

- 13 Reimbursement, if any, to EPO deposit account number**

2800.0321

Account holder's name

VOSSIUS & PARTNER

- 13. Remboursements éventuels à effectuer sur le compte courant ouvert auprès de l'OEB numéro**

Nom du titulaire du compte

- 14. Unterschrift(en) des (der) Anmelders(s) oder Vertreters**

Ort / Datum

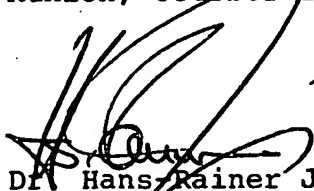
- 14. Signature(s) of applicant(s) or representative**

Place / Date

Munich, October 13, 1998

- 14. Signature(s) du (des) demandeur(s) ou mandataire**

Lieu / Date


Dr. Hans-Rainer Jaenichen
European Patent Attorney

Für Angestellte (Art. 133(3) EPÜ) mit allgemeiner Vollmacht:

Nr. _____

Name(n) des (der) Unterzeichneten bitte mit Schreibmaschine wiederholen. Bei juristischen Personen bitte auch die Stellung des (der) Unterzeichneten innerhalb der Gesellschaft eintragen

For employees (Art. 133(3) EPC) having a general authorisation:

No. _____

Please type name(s) under signature(s). In the case of legal persons, the position of the signatory within the company should also be typed

Pour les employés (art. 133(3) CBE) disposant d'un pouvoir général:

N° _____

Veuillez faire figurer le nom dactylographié sous la signature. Si ce nom désigne une personne morale, ajoutez la mention dactylographiée de la position occupée par le signataire au sein de la société

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72.041.12-3

2. The polynucleotide of claim 1 which is DNA or RNA.
3. The DNA of claim 2 which is genomic DNA.

4. The polynucleotide of any one of claims 1 to 3 which is fused to a heterologous polynucleotide.
5. A vector containing the polynucleotide of any one of claims 1 to 4.
6. The vector of claim 5 in which the polynucleotide is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic host cells.
7. A host cell genetically engineered with the polynucleotide of any one of claims 1 to 4 or the vector of claim 5 or 6.
8. A process for producing a polypeptide having TNF delta or TNF epsilon activity comprising: culturing the host cell of claim 7 and recovering the polypeptide encoded by said polynucleotide from the culture.
9. A process for producing cells capable of expressing a polypeptide having TNF delta or TNF epsilon activity comprising genetically engineering cells with the vector of claim 5 or 6.
10. A polypeptide having the amino acid sequence encoded by a polynucleotide of any one of claims 1 to 4 or obtainable by the process of claim 8.
11. An antibody against the polypeptide of claim 10.
12. A nucleic acid molecule which specifically hybridizes to a polynucleotide of any one of claims 1 to 4.
13. An antagonist/inhibitor of the polypeptide of claim 10.
14. A pharmaceutical composition comprising the polynucleotide of any one of claims 1 to 4, the polypeptide of claim 10 or a DNA encoding and capable of expressing said polypeptide in vivo or the antagonist/inhibitor of claim 13 and optionally a pharmaceutically acceptable carrier.

15. A diagnostic composition comprising the polynucleotide of any one of claims 1 to 4, the nucleic acid molecule of claim 12 or the antibody of claim 11.
16. Use of the polypeptide of claim 10 or the polynucleotide of any one of claims 1 to 4 for the preparation of a pharmaceutical composition for the treatment of neoplasia, for wound-healing, for the treatment of restenosis, for regulating hematopoiesis in endothelial cell development, for stimulating an immune response against parasitic, bacterial or viral infections, or for the treatment and/or prevention of autoimmune diseases.
17. Use of the antagonist/inhibitor of claim 13 for the preparation of a pharmaceutical composition for the treatment of cachexia, cerebral malaria, rheumatoid arthritis, for the prevention of graft-host rejection, for inhibiting bone resorption, for the treatment and/or prevention of osteoporosis, or for the treatment of endotoxic shock.
18. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 10 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
19. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 10 in a sample derived from a host.
20. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 10 comprising:
 - (a) contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF delta polypeptide and a compound under conditions to permit binding to the receptor; and

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- (b) determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.
21. A method for the production of a pharmaceutical composition comprising the steps of the method of claim 20 and (c) formulating the compound identified in step (b) in a pharmaceutically acceptable form.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

13. Okt. 1998

(i) APPLICANT: NI, JIAN
YU, GUO-LIANG
GENTZ, REINER
DILLON, PATRICK

(ii) TITLE OF INVENTION: HUMAN TUMOR NECROSIS FACTOR DELTA AND
EPSILON

(iii) NUMBER OF SEQUENCES: 10

(iv) CORRESPONDENCE ADDRESS:

(A) ADDRESSEE: HUMAN GENOME SCIENCES, INC.
(B) STREET: 9410 KEY WEST AVENUE
(C) CITY: ROCKVILLE
(D) STATE: MD
(E) COUNTRY: US
(F) ZIP: 20850

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: A. ANDERS BROOKES
(B) REGISTRATION NUMBER: 36,373
(C) REFERENCE/DOCKET NUMBER: PF253

(ix) TELECOMMUNICATION INFORMATION:

(A) TELEPHONE: (301) 309-8504
(B) TELEFAX: (301) 309-8512

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1717 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 333..1031

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

M 13.10.98

TCTCAAGACA ACGCTGTCCC CACGACGGAG TGCCAGGAGC ACTAACAGTA CCCTTAGATT	120
GCTTTCCTCC TCCCTCCTTT TTTATTTTCA AGTTCCTTTT TATTTCTCCT TGC GTAACAA	180
CCTTCTTCCC TTCTGCACCA CTGCCCCTAC CCTTACCCGC GCCGCCACCT CCTTGCTACA	240
CCACTCTTGA AACCACAGCT GTTGGCAGGG TCCCCCAGCT CATGCCAGCC TCATCTCCTT	300
TCTTGCTAGC CCCCAAAGGG CCTCCAGGCA AC ATG GGG GGC CCA GTC AGA GAG	353
Met Gly Gly Pro Val Arg Glu	
1 5	
CCG GCA CTC TCA GTT GCC CTC TGG TTG AGT TGG GGG GCA GCT CTG GGG	401
Pro Ala Leu Ser Val Ala Leu Trp Leu Ser Trp Gly Ala Ala Leu Gly	
10 15 20	
GCC GTG GCT TGT GCC ATG GCT CTG CTG ACC CAA CAA ACA GAG CTG CAG	449
Ala Val Ala Cys Ala Met Ala Leu Leu Thr Gln Gln Thr Glu Leu Gln	
25 30 35	
AGC CTC AGG AGA GAG GTG AGC CGG CTG CAG AGG ACA GGA GGC CCC TCC	497
Ser Leu Arg Arg Glu Val Ser Arg Leu Gln Arg Thr Gly Gly Pro Ser	
40 45 50 55	
CAG AAT GGG GAA GGG TAT CCC TGG CAG AGT CTC CCG GAG CAG AGT TCC	545
Gln Asn Gly Glu Gly Tyr Pro Trp Gln Ser Leu Pro Glu Gln Ser Ser	
60 65 70	
GAT GCC CTG GAA GCC TGG GAG AAT GGG GAG AGA TCC CGG AAA AGG AGA	593
Asp Ala Leu Glu Ala Trp Glu Asn Gly Glu Arg Ser Arg Lys Arg Arg	
75 80 85	
GCA GTG CTC ACC CAA AAA CAG AAG AAG CAG CAC TCT GTC CTG CAC CTG	641
Ala Val Leu Thr Gln Lys Gln Lys Lys Gln His Ser Val Leu His Leu	
90 95 100	
GTT CCC ATT AAC GCC ACC TCC AAG GAT GAC TCC GAT GTG ACA GAG GTG	689
Val Pro Ile Asn Ala Thr Ser Lys Asp Asp Ser Asp Val Thr Glu Val	
105 110 115	
ATG TGG CAA CCA GCT CTT AGG CGT GGG AGA GGC CTA CAG GCC CAA GGA	737
Met Trp Gln Pro Ala Leu Arg Arg Gly Arg Gly Leu Gln Ala Gln Gly	
120 125 130 135	
TAT GGT GTC CGA ATC CAG GAT GCT GGA GTT TAT CTG CTG TAT AGC CAG	785
Tyr Gly Val Arg Ile Gln Asp Ala Gly Val Tyr Leu Leu Tyr Ser Gln	
140 145 150	
GTC CTG TTT CAA GAC GTG ACT TTC ACC ATG GGT CAG GTG GTG TCT CGA	833
Val Leu Phe Gln Asp Val Thr Phe Thr Met Gly Gln Val Val Ser Arg	
155 160 165	
GAA GGC CAA GGA AGG CAG GAG ACT CTA TTC CGA TGT ATA AGA AGT ATG	881
Glu Gly Gln Gly Arg Gln Glu Thr Leu Phe Arg Cys Ile Arg Ser Met	
170 175 180	
CCC TCC CAC CCG GAC CGG GCC TAC AAC AGC TGC TAT AGC GCA GGT GTC	929
Pro Ser His Pro Asp Arg Ala Tyr Asn Ser Cys Tyr Ser Ala Gly Val	
185 190 195	
TTC CAT TTA CAC CAA GGG GAT ATT CTG AGT GTC ATA ATT CCC CGG GCA	977
Phe His Leu His Gln Gly Asp Ile Leu Ser Val Ile Ile Pro Arg Ala	

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200	205	210	215	
AGG GCG AAA CTT AAC CTC TCT CCA CAT GGA ACC TTC CTG GGG TTT GTG				1025
Arg Ala Lys Leu Asn Leu Ser Pro His Gly Thr Phe Leu Gly Phe Val				
	220	225	230	
AAA CTG TGATTGTGTT ATAAAAAGTG GCTCCCAGCT TGGAAGACCA GGGTGGGTAC				1081
Lys Leu				
ATACTGGAGA CAGCCAAGAG CTGAGTATAT AAAGGAGAGG GAATGTGCAG GAACAGAGGC				1141
GTCTTCCTGG GTTTGGCTCC CCGTTCCTCA CTTTTCCCTT TTCATTCCCA CCCCCTAGAC				1201
TTTGATTTTA CGGATATCTT GCTTCTGTTC CCCATGGAGC TCCGAATTCT TCGTGTGTG				1261
TAGATGAGGG GCGGGGGACG GCGCCAGGC ATTGTCCAGA CCTGGTCGGG GCCCACTGGA				1321
AGCATCCAGA ACAGCACCAC CATCTAGCGG CCGCTCTAGA GGATCCCTCG AGGGGCCCAA				1381
GCTTACGCGT GCATGCGACG TCATAGCTCT CTCCCTATAG TGAGTCGTAT TATAAGCTAG				1441
CTTGGGATCT TTGTGAAGGA ACCTTACTTC TGTGGTGTGA CATAATTGGA CAACTACCT				1501
ACAGAGATTT AAAGCTCTAA GGTAATATA AAATTTTAA GTGTATAATG TGTTAACTA				1561
GCTGCATATG CTTGCTGCTT GAGAGTTTGG CTTACTGAGT ATGATTATGA AAATATTATA				1621
CACAGGAGCT AGTGATCTAT GTTGGTTTAA GATCAAGCCA AGGTCATTCA GGCCTCAGCT				1681
CAAGCTGTCA TGATCATATC AGCATACAAT TGTGAG				1717

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Gly Gly Pro Val Arg Glu Pro Ala Leu Ser Val Ala Leu Trp Leu			
1	5	10	15
Ser Trp Gly Ala Ala Leu Gly Ala Val Ala Cys Ala Met Ala Leu Leu			
	20	25	30
Thr Gln Gln Thr Glu Leu Gln Ser Leu Arg Arg Glu Val Ser Arg Leu			
	35	40	45
Gln Arg Thr Gly Gly Pro Ser Gln Asn Gly Glu Gly Tyr Pro Trp Gln			
	50	55	60
Ser Leu Pro Glu Gln Ser Ser Asp Ala Leu Glu Ala Trp Glu Asn Gly			
	65	70	75
Glu Arg Ser Arg Lys Arg Arg Ala Val Leu Thr Gln Lys Gln Lys Lys			
	85	90	95
Gln His Ser Val Leu His Leu Val Pro Ile Asn Ala Thr Ser Lys Asp			

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100

105

110

Asp Ser Asp Val Thr Glu Val Met Trp Gln Pro Ala Leu Arg Arg Gly
 115 120 125
 Arg Gly Leu Gln Ala Gln Gly Tyr Gly Val Arg Ile Gln Asp Ala Gly
 130 135 140
 Val Tyr Leu Leu Tyr Ser Gln Val Leu Phe Gln Asp Val Thr Phe Thr
 145 150 155 160
 Met Gly Gln Val Val Ser Arg Glu Gly Gln Gly Arg Gln Glu Thr Leu
 165 170 175
 Phe Arg Cys Ile Arg Ser Met Pro Ser His Pro Asp Arg Ala Tyr Asn
 180 185 190
 Ser Cys Tyr Ser Ala Gly Val Phe His Leu His Gln Gly Asp Ile Leu
 195 200 205
 Ser Val Ile Ile Pro Arg Ala Arg Ala Lys Leu Asn Leu Ser Pro His
 210 215 220
 Gly Thr Phe Leu Gly Phe Val Lys Leu
 225 230

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1305 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2..505

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

G GGG ACA GGA GGC CCC TCC CAG AAT GGG GAA GGG TAT CCC TGG CAG	46
Gly Thr Gly Gly Pro Ser Gln Asn Gly Glu Gly Tyr Pro Trp Gln	
1 5 10 15	
AGT CTC CCG GAG CAG AGT TCC GAT GCC CTG GAA GCC TGG GAG AGT GGG	94
Ser Leu Pro Glu Gln Ser Ser Asp Ala Leu Glu Ala Trp Glu Ser Gly	
20 25 30	
GAG AGA TCC CGG AAA AGG AGA GCA GTG CTC ACC CAA AAA CAG AAG AAT	142
Glu Arg Ser Arg Lys Arg Arg Ala Val Leu Thr Gln Lys Gln Lys Asn	
35 40 45	
GAC TCC GAT GTG ACA GAG GTG ATG TGG CAA CCA GCT CTT AGG CGT GGG	190
Asp Ser Asp Val Thr Glu Val Met Trp Gln Pro Ala Leu Arg Arg Gly	
50 55 60	
AGA GGC CTA CAG GCC CAA GGA TAT GGT GTC CGA ATC CAG GAT GCT GGA	238
Arg Gly Leu Gln Ala Gln Gly Tyr Gly Val Arg Ile Gln Asp Ala Gly	
65 70 75	

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GTT TAT CTC CTG TAT AGC CAG GTC CTG TTT CAA GAC GTG ACT TTC ACC	286
Val Tyr Leu Leu Tyr Ser Gln Val Leu Phe Gln Asp Val Thr Phe Thr	
80 85 90 95	
ATG GGT CAG GTG GTG TCT CGA GAA GGC CAA GGA AGG CAG GAG ACT CTA	334
Met Gly Gln Val Val Ser Arg Glu Gly Gln Gly Arg Gln Glu Thr Leu	
100 105 110	
TTC CGA TGT ATA AGA AGT ATG CCC TCC CAC CCG GAC CGG GCC TAC AAC	382
Phe Arg Cys Ile Arg Ser Met Pro Ser His Pro Asp Arg Ala Tyr Asn	
115 120 125	
AGC TGC TAT AGC GCA GGT GTC TTC CAT TTA CAC CAA GGG GAT ATT CTG	430
Ser Cys Tyr Ser Ala Gly Val Phe His Leu His Gln Gly Asp Ile Leu	
130 135 140	
AGT GTC ATA ATT CCC CGG GCA AGG GCG AAA CTT AAC CTC TCT CCA CAT	478
Ser Val Ile Ile Pro Arg Ala Arg Ala Lys Leu Asn Leu Ser Pro His	
145 150 155	
GGA ACC TTC CTG GGG TTT GTG AAA CTG TGATTGTGTT ATAAAAAGTG	525
Gly Thr Phe Leu Gly Phe Val Lys Leu	
160 165	
GCTCCCAGCT TGGAAGACCA GGGTGGGTAC ATACTGGAGA CAGCCAAGAG CTGAGTATAT	585
AAAGGAGAGG GAATGTGCAG GAACAGAGGC GTCTTCCTGG GTTTGGCTCC CCGTTCCTCA	645
CTTTTCCCTT TTCATTCCCA CCCCTAGAC TTTGGATTTT ACGGATATCT TGCTTCTGTT	705
CCCCATGGAG CTCCGAATTC TTGCGTGTGT GTAGATGAGG GGCGGGGGAC GGGCGCCAGG	765
CATTGTTTCAG ACCTGGTCGG GGCCCACTGG AAGCATCCAG AACAGCACCA CCATCTAGCG	825
GCGCTCGAGG GAAGCACCGC GGGTTGGCCG AAGTCCACGA AGCCGCCTCT GCTAGGGAAA	885
ACCCTGGTTC TCCATGCCAC AACTCTCTCC AGGGTGGCCT CTGCCTCTTC AACCCACAA	945
AGAAGCCTTA ACCTACGTCC TTCTCTCCAT CTATCGGACC CCAGTTTCCA TCACTATCTC	1005
CAGAGATGTA GCTATTATGC GCCCGTCTAC AGGGGGTGCC CGACGATGAC GGTGCCTTCG	1065
CAGTCAAATT ACTCTTCGGG TCCCAAGGTT TGGCTTTCAC GCGCTCCATT GCCCCGGCGT	1125
GGCAGGCCAT TCCAAGCCCT TCCGGGCTGG AACTGGTGTC GGAGGAGCCT CGGGTGTATC	1185
GTACGCCCTG GTGTTGGTGT TGCCTCACTC CTCTGAGCTC TTCTTTCTGA TCAAGCCCTG	1245
CTTAAAGTTA AATAAAATAG AATGAATGAT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA	1305

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 168 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

M 13.10.98

Gly Thr Gly Gly Pro Ser Gln Asn Gly Glu Gly Tyr Pro Trp Gln Ser
1 5 10 15

Leu Pro Glu Gln Ser Ser Asp Ala Leu Glu Ala Trp Glu Ser Gly Glu
20 25 30

Arg Ser Arg Lys Arg Arg Ala Val Leu Thr Gln Lys Gln Lys Asn Asp
35 40 45

Ser Asp Val Thr Glu Val Met Trp Gln Pro Ala Leu Arg Arg Gly Arg
50 55 60

Gly Leu Gln Ala Gln Gly Tyr Gly Val Arg Ile Gln Asp Ala Gly Val
65 70 75 80

Tyr Leu Leu Tyr Ser Gln Val Leu Phe Gln Asp Val Thr Phe Thr Met
85 90 95

Gly Gln Val Val Ser Arg Glu Gly Gln Gly Arg Gln Glu Thr Leu Phe
100 105 110

Arg Cys Ile Arg Ser Met Pro Ser His Pro Asp Arg Ala Tyr Asn Ser
115 120 125

Cys Tyr Ser Ala Gly Val Phe His Leu His Gln Gly Asp Ile Leu Ser
130 135 140

Val Ile Ile Pro Arg Ala Arg Ala Lys Leu Asn Leu Ser Pro His Gly
145 150 155 160

Thr Phe Leu Gly Phe Val Lys Leu
165

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 231 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Pro Ile Pro Met Ile Pro Asp Val Glu Leu Ala Glu Glu Ala Leu
1 5 10 15

Pro Lys Lys Thr Gly Gly Pro Gln Gly Ser Arg Arg Cys Leu Phe Leu
20 25 30

Ser Leu Phe Ser Phe Leu Ile Val Ala Gly Ala Thr Thr Leu Phe Cys
35 40 45

Leu Leu His Phe Gly Val Ile Gly Pro Gln Arg Glu Glu Ser Pro Arg
50 55 60

Asp Leu Ser Leu Met Ser Leu Leu Ala Gln Ala Arg Ser Ser Ser Arg
65 70 75 80

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Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro Gln Ala
 85 90 95
 Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala
 100 105 110
 Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly
 115 120 125
 Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro
 130 135 140
 Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser
 145 150 155 160
 Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
 165 170 175
 Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile
 180 185 190
 Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala
 195 200 205
 Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val
 210 215 220
 Tyr Phe Gly Ile Ile Ala Leu
 225 230

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 204 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Thr Pro Pro Glu Arg Leu Phe Leu Pro Arg Val Cys Gly Thr Thr
 1 5 10 15
 Leu His Leu Leu Leu Leu Gly Leu Leu Leu Val Leu Ile Pro Gly Ala
 20 25 30
 Gln Gly Leu Pro Gly Val Gly Leu Thr Pro Ser Ala Ala Cys Thr Ala
 35 40 45
 Arg Gln His Pro Lys Met His Leu Ala His Ser Thr Leu Lys Pro Ala
 50 55 60
 Ala His Leu Ile Gly Asp Pro Ser Lys Gln Asn Ser Leu Leu Trp Arg
 65 70 75 80
 Ala Asn Thr Asp Arg Ala Phe Leu Gln Asp Gly Phe Ser Leu Ser Asn
 85 90 95

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Asn Ser Leu Leu Val Pro Thr Ser Gly Ile Tyr Phe Val Tyr Ser Gln
 100 105 110
 Val Val Phe Ser Gly Lys Ala Tyr Ser Pro Lys Ala Pro Ser Pro Leu
 115 120 125
 Tyr Leu Ala His Glu Val Gln Leu Phe Ser Ser Gln Tyr Pro Phe His
 130 135 140
 Val Pro Leu Leu Ser Ser Gln Lys Met Val Tyr Pro Gly Leu Gln Glu
 145 150 155 160
 Pro Trp Leu His Ser Met Tyr His Gly Ala Ala Phe Gln Leu Thr Gln
 165 170 175
 Gly Asp Gln Leu Ser Thr His Thr Asp Gly Ile Pro His Leu Val Leu
 180 185 190
 Ser Pro Ser Thr Val Phe Phe Gly Ala Phe Ala Leu
 195 200

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGGATCCC AGAGCCTCAC CACAG

25

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGCAAGCTTA CAATCACAGT TTCACAAAC

29

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GCGGGATCCC CAGAGCCTCA CCACAG

26

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 29 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGCTCTAGAA CAATCACAGT TTCACAAAC

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PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

10 08. 1998

PCT Article 36 and Rule 70)

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Applicant's or agent's file reference PF253PCT	FOR FURTHER ACTION See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)	
International application No. PCT/US96/03774	International filing date (day/month/year) 14 MARCH 1996	Priority date (day/month/year) NONE
International Patent Classification (IPC) or national classification and IPC Please See Supplemental Sheet.		
Applicant HUMAN GENOME SCIENCES, INC.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 6 sheets.

☐ This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority. (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of — sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☐ Priority
- III ☒ Non-establishment of report with regard to novelty, inventive step or industrial applicability
- IV ☐ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☐ Certain documents cited
- VII ☐ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Bob Verboom 14. 8. 1998

Date of submission of the demand 14 OCTOBER 1997	Date of completion of this report 01 JULY 1998
Name and mailing address of the IPEA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DARYL A. BASHAM
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/03774

I. Basis of the report

1. This report has been drawn on the basis of *(Substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments):*

- ☒ the international application as originally filed.
- ☒ the description, pages 1-64 , as originally filed.
pages NONE , filed with the demand.
pages NONE , filed with the letter of _____.
pages _____ , filed with the letter of _____.
- ☒ the claims, Nos. 1-30 , as originally filed.
Nos. NONE , as amended under Article 19.
Nos. NONE , filed with the demand.
Nos. NONE , filed with the letter of _____.
Nos. _____ , filed with the letter of _____.
- ☒ the drawings, sheets/fig 1-10 , as originally filed.
sheets/fig NONE , filed with the demand.
sheets/fig NONE , filed with the letter of _____.
sheets/fig _____ , filed with the letter of _____.

2. The amendments have resulted in the cancellation of:

- ☒ the description, pages NONE .
- ☒ the claims, Nos. NONE .
- ☒ the drawings, sheets/fig NONE .

3. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box Additional observations below (Rule 70.2(c)).

4. Additional observations, if necessary:

NONE

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/03774

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

The question whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been and will not be examined in respect of:

☐ the entire international application.

☒ claims Nos. 21-30

because:

☐ the said international application, or the said claim Nos. _ relate to the following subject matter which does not require international preliminary examination (*specify*).

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. _ are so unclear that no meaningful opinion could be formed (*specify*).

☐ the claims, or said claims Nos. _ are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for said claims Nos. 21-30.

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/03774

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**1. STATEMENT**

Novelty (N)	Claims <u>5-12 and 16-20</u>	YES
	Claims <u>1-4 and 13-15</u>	NO
Inventive Step (IS)	Claims <u>5-12 and 16-20</u>	YES
	Claims <u>1-4 and 13-15</u>	NO
Industrial Applicability (IA)	Claims <u>1-20</u>	YES
	Claims <u>NONE</u>	NO

2. CITATIONS AND EXPLANATIONS

Claims 1-3, 13-15 lack novelty under PCT Article 33(2) as being anticipated by ADAMS, et al.

ADAMS et al teach a cDNA clone which comprises at least 15 bases of the polynucleotide encoding a polypeptide comprising an amino acid sequence of instant SEQ ID NO: 2 (compare nucleotides 926-942 of ADAMS et al with nucleotides 407-424 of the instant SEQ ID NO: 1), including RNA (which is anticipated by cDNA), and as the sequence was cloned, vectors and host cells comprising are likewise anticipated (at page 634, Table 2 and 632, ABSTRACT). Therefore, ADAMS et al teach all of the material compositions of instant claims 1-3 and 13-15 because the sequences of ADAMS et al are contained within the coding region of SEQ ID NO: 1, and as the length/sequence identity limitations are met by the recited nucleic acid array of ADAMS et al, its complement is contemplated.

Claims 1-4 and 13-15 lack novelty under PCT Article 33(2) as being anticipated by ALLET, et al.

ALLET et al teach a cDNA clone (column 15, Example 4) which comprises 12 (at least 11) nucleotides of a polynucleotide which encodes a polypeptide comprising an amino acid sequence of instant SEQ ID NO: 2 (compare amino acids 113-116 of ALLET et al with amino acids 30-32 of the instant Figure 1A, which is instant SEQ ID NO: 2), including RNA (which is anticipated by cDNA), genomic DNA (at column 4, lines 65-67; the "gene" definition would include genomic DNA), vectors, host cells and a means of producing the TNF recombinantly (claims 9-11). Therefore, ALLET et al teach all of the material compositions of claims 1-4 and 13-15 because, as embraced polynucleotides need only be 70% identical to 15 nucleotides of any degenerate sequence encoding the same amino acids, only 11 nucleotides are required (less than 3 codons) to meet the limitations of the claims.

Claims 5-12 and 16-20 the criteria set out in PCT Article 33(2)-(3), because the prior art does not teach or fairly suggest polypeptides comprising the enumerated sequences nor (Continued on Supplemental Sheet.)

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

The description fails to adequately enable practice of the claimed invention because:

By claiming polynucleotide sequences using a polypeptide as the reference molecule, all degenerate polynucleotides capable of encoding the amino acid sequences are embraced. If, for example, all of the third positions of the codons are replaceable by wobble hypothesis rules (given nucleic acids of equal length) to give the same amino acid sequence by degeneracy of the genetic code, a nucleic acid having approximately 66.6% identity to the naturally occurring sequence would be expected to be present in the population of molecules. However, a polynucleotide which is 70% identical to said perfectly degenerate polynucleotide (having 66.6% identity) would not encode the same polypeptide and neither would such a molecule serve as a probe for the isolation of related proteins nor could it be used to produce the polypeptide as set forth in the instant SEQ ID NO: 6 by recombinant means (the overall homology could be as low as 47%). As such, the limitations of the claims embrace polynucleotides which encode polypeptides that are not envisaged by the description. Therefore, as the descriptions fails to adequately provide guidance or examples of a polynucleotide having 47% identity with the naturally occurring polynucleotide as set forth in SEQ ID NO: 1, one skilled in the art could not make or use the embraced polynucleotides without undue experimentation because such a polynucleotide would not have a predictable (assayable) function.

Claims 1-4, 9 and 13-15 are not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

The description fails to adequately enable practice of the claimed invention because:

As written, claims embrace polypeptides that have approximately 58 and 56 amino acid residues randomly substituted in SEQ ID NOS: 2 and 4, respectively (at least 70% homology). While one skilled in the art would be able to make such random changes in sequence mechanically, there is no disclosed rationale that guides one to make changes to generate variants having 70% identity in the absence of a confirmation of the possession of said variant(s) of a biological property peculiar to TNF, and such a functional limitation is not apparent in the claims. Moreover, it is well known in the art that specific amino acids are essential for proper protein folding such that the substitution of amino acid residues cannot be made indiscriminately given the potential negative effects caused by (Continued on Supplemental Sheet.)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No.

PCT/US96/03774

Supplemental Box

(To be used when the space in any of the preceding boxes is not sufficient)

Continuation of: Boxes I - VIII

Sheet 10

CLASSIFICATION:

The International Patent Classification (IPC) and/or the National classification are as listed below:

IPC(6): C07H 21/02, 21/04; C07K 1/00, 14/00; C12N 5/00, 15/09, 15/63 and US Cl.: 536/23.1, 23.51; 530/350; 435/172.3, 252.3, 320.1, 814; 935/1, 3, 23, 60

V. 2. REASONED STATEMENTS - CITATIONS AND EXPLANATIONS (Continued):

polynucleotides encoding said sequences.

Claims 1-20 meet the criteria set out in PCT Article 33(4) as having industrial applicability.

NEW CITATIONS

GEORGE et al. Current Methods in Sequence Comparison and Analysis. In: Macromolecular Sequencing and Synthesis: Selected Methods and Applications. Edited by D. H. Schlesinger. New York: Alan R. Liss, Inc. 1998, pages 127-149.

VIII. CERTAIN OBSERVATIONS ON THE APPLICATION (Continued):

perturbations in amino acid sequence. In the absence of function information peculiar to TNF, a person of ordinary skill in the art would be unable to make and use a functional polypeptide that is at least 70% identical to the polypeptide as set forth in SEQ ID NO: 2 and 4 without undue experimentation because selection of mutable sites in the amino acid sequence based on the description would be arbitrary resulting in the analysis of an inordinate number of non-functional variants.

Claim 17 is not enabled as required under PCT Rule 5.1(a) for the reasons set forth in the immediately preceding paragraph.

Claims 1-4, 9, and 13-17 objected to under PCT Rule 66.2(a)(v) as lacking clarity under PCT Article 6 because the claims are indefinite for the following reason(s):

Claims 1-4, 9 and 13-17 recite "...% identity". This phrase has unambiguous meaning when it is applied to the comparison of two sequences of equal length, however, sequences of unequal length are evidently considered to be comparable by this standard. It is not clear as to how gaps are to be assessed in determining identity where gaps are required to optimally align two sequences of unequal length. This ambiguity may be demonstrated by the following examples: consider two sequences, ABCDEF and ABEP. These could be compared in four ways:

ABCDEF 4/6 = 67%

AB-EP 4/4 = 100%

ABCDEF

2/6 = 33%

ABEP

2/4 = 50%

In the absence of a disclosure of the algorithm by which "...% identity" is to be determined, the claims can only be considered definite if comparisons are limited to sequences of identical length. To illustrate this issue, the Examiner has cited George, et al. (1988) which teaches that "the results of the analysis are entirely dependent on the choice of scoring rules" (page 130, column 2, lines 4-6). It is apparent that an algorithm is required to determine the "% identity".



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Carella, Byrne, Bain, Gilfillan, Cecchi, Stewart &
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6 Becker Farm Road;
Roseland, NJ 07068

ETATS-UNIS D'AMERIQUE

Datum/Date

02/10/97

Zeichen/Ref /Réf

Anmeldung Nr./Application No./Demande n° //Patent Nr./Patent No./Brevet n°

96910483.5 - -PCT/US9603774

Anmelder/Applicant/Demandeur//Patentinhaber/Proprietor/Titulaire

HUMAN GENOME SCIENCES, INC.

NOTE: The following information concerns the steps which you are required to take for entry into the regional phase before the EPO. You are strongly advised to read it carefully. Failure to take the appropriate steps in due time could lead to the application being deemed withdrawn.

1. European patent application no. 96910483.5 has been allotted to the above-mentioned international patent application.
2. Applicants having neither a residence nor their principal place of business within the territory of one of the EPC Contracting States may initiate the regional (European) processing of the international application themselves, provided they do so before expiry of the 21st or 31st month as from the priority date (see Legal Advice of the EPO no. 18/92 published in OJ EPO 1992, 58).

Note, however, that such applicants must be represented in the regional phase before the EPO as designated or elected Office by a professional representative whose name appears on the EPO list of representatives (Arts. 133(2) and 134(1) EPC).

After expiry of the 21st or 31st month, any procedural steps which are taken by the representative of the applicant in the international phase, who is not, however, entitled to practise before the EPO, will have no effect and will, thus, result in loss of rights.

The appointment of a professional representative entitled to practise before the EPO is possible/advisable at an early stage during the international phase (any time after the 14th month from the priority date) in view of representing applicants before the EPO as designated or elected Office.

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Therefore, an appointment in due time is strongly recommended, if it is intended that this representative should already act for entry into the regional phase, otherwise all communications will be forwarded from the EPO directly to the applicant.

3. Applicants having their address within the territory of one of the EPC Contracting States are not obliged to appoint a professional representative entitled to practise before the EPO to represent them in the regional phase where the EPO is designated or elected Office.

Note that due to the complexity of the proceedings, applicants are strongly advised to appoint such representative. Please keep in mind that, if a professional representative before the EPO has already acted for the applicant during the international phase, this representative is not automatically regarded as the representative for the regional phase.

4. Applicants and professional representatives are recommended to file EPO Form 1200 (available free of charge from the EPO) for entry into the regional phase. The use of Form 1200, however, is not mandatory.
5. FOR ENTRY INTO THE REGIONAL PHASE BEFORE THE EPO the following procedural steps must be taken. (Note that non-completion or ineffective completion of the required steps will result in loss of rights or other disadvantage.)

5.1 Within 21 months from the date of filing or (where applicable) from the earliest priority date if the EPO acts as DESIGNATED OFFICE pursuant to Article 22(1) PCT:

- a) Filing of a translation of the international application in an EPO official language if the International Bureau did not publish the application in one of those languages (Art. 22(1) PCT and Rule 104b(1)(a) EPC).

Note that if such translation is not filed in due time, the international application before the EPO is deemed withdrawn (Art. 24(1)(iii) PCT).

- b) Payment of the national fee [national basic fee, the designation fee for each State designated, (where applicable) the claims fees for the eleventh and each subsequent claim] and the search fee, where a supplementary European search report has to be drawn up (Rule 104b(1)(b), (c) EPC).

Upon expiry of the 21-month time limit provided for in Rule 104b(1) EPC the EPO sends the applicant or his appointed professional representative the communication pursuant to Rule 85a(1) EPC (Form 1217) and (where applicable) Rule 69(1) EPC (Form 1205)

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unless it has been notified of its designation as elected Office in due time.

5.2 Within 31 months from the date of filing or (where applicable) from the earliest priority date if the EPO acts as ELECTED OFFICE pursuant to Article 39(1)(a) PCT:

a) Filing of a translation as under 5.1 a).

b) Payment of the fees as under 5.1 b).

c) Filing of the written request for examination and payment of the examination fee (Rule 104b(1)(d) EPC).

Note that both acts must be performed in due time, otherwise the European patent application shall be deemed to be withdrawn (Art. 94(3) EPC).

d) Payment of the renewal fee for the third year, if due before the expiration of the 31-month term (Rule 104b(1)(e) EPC).

6. The amounts of the fees (equivalent in all currencies) are regularly published in the Official Journal of the EPO.

If the national basic fee, the designation fees or the search fee have not been paid in time, they may still be validly paid within a grace period of one month as from notification of an EPO communication (Rule 85a(1) EPC).

If the renewal fee is not paid in time, it may still be validly paid within six months from the due date (Art. 86(2) EPC).

In both cases, a surcharge is due.

7. The international search report under Article 18 PCT (or the declaration under Article 17(2)(a) PCT) has been published by the International Bureau. The date of publication can be ascertained from the copy of the published application documents sent by the International Bureau or from the international search report, if published separately. This publication takes the place of the mention of the publication of the European search report (Art. 157(1) EPC).

A request for examination, comprising a written request and payment of the examination fee, must be filed up to the end of six months after the above date.



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However, in view of Article 22 or 39 PCT in conjunction with Rule 104b(1)(d) EPC, the period for filing the request for examination does not expire before 21 or 31 months, respectively, from the date of filing (where applicable, the earliest priority date).

A period of grace of one month from notification of an EPO communication is available in case either or both of the above acts have not been performed in time. Accordingly, a surcharge is due (Rule 85b EPC).

8. This information letter is addressed by the EPO to the agent, if any, having acted for the applicant during the international phase of the application.

Any further notifications on procedural matters will be addressed to the applicant, respectively his European representative, if the appointment of the latter has been communicated to the EPO in due time. In case of non-resident applicants, notification shall be deemed to have been made when dispatch has taken place, even if the letter is returned to the sender owing to the impossibility of delivering it to the addressee (Rule 78(2) EPC).

9. For further details see the information for PCT applicants concerning time limits and procedural steps before the EPO as a designated and as an elected Office under the PCT (published as Supplement No. 1 to OJ EPO 12/1992, with changes published in OJ EPO 1994, 131).

RECEIVING SECTION



Anmeldung Nr./Application No./Demande n°/Patent Nr./Patent No./Brevet n°

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(54) Title: HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON (57) Abstract The invention relates to human TNF delta and TNF epsilon polypeptides, polynucleotides encoding the polypeptides, methods for producing the polypeptides, in particular by expressing the polynucleotides, and agonists and antagonists of the polypeptides. The invention further relates to methods for utilizing such polynucleotides, polypeptides, agonists and antagonists for applications, which relate, in part, to research, diagnostic and clinical arts.		

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HUMAN TUMOR NECROSIS FACTOR DELTA AND EPSILON

This invention relates, in part, to newly identified polynucleotides and polypeptides; variants and derivatives of the polynucleotides and polypeptides; processes for making the polynucleotides and the polypeptides, and their variants and derivatives; agonists and antagonists of the polypeptides; and uses of the polynucleotides, polypeptides, variants, derivatives, agonists and antagonists. In particular, in these and in other regards, the invention relates to polynucleotides and polypeptides of human tumor necrosis factor delta and epsilon, sometimes hereinafter referred to as "TNF delta" and "TNF epsilon".

BACKGROUND OF THE INVENTION

Human tumor necrosis factors α (TNF- α) and β (TNF- β or lymphotoxin) are related members of a broad class of polypeptide mediators, which includes the interferons, interleukins and growth factors, collectively called cytokines (Beutler, B. and Cerami, A., *Annu. Rev. Immunol.*, 7:625-655, 1989).

Tumor necrosis factor (TNF- α and TNF- β) was originally discovered as a result of its anti-tumor activity, however, now it is recognized as a pleiotropic cytokine capable of numerous biological activities including apoptosis of some transformed cell lines, mediation of cell activation and proliferation and also as playing important roles in immune regulation and inflammation.

To date, there are nine known members of the TNF-ligand superfamily, TNF- α , TNF- β (lymphatoxin- α), LT- β , OX40L, FASL, CD30L, CD27L, CD40L and 4-1BBL. The ligands of the TNF ligand superfamily are acidic, TNF-like molecules with approximately 20% sequence homology in the extracellular domains (range, 12%-36%) and exist mainly as membrane-bound forms with the biologically active form being a trimeric/multimeric complex. Soluble forms of the TNF ligand superfamily have only been identified so far for TNF, LT α , and FASL (for a general review, see Gruss, H. and Dower, S.K., *Blood*, 85 (12):3378-3404 (1995)), which is hereby incorporated by reference in its entirety.

These proteins are involved in regulation of cell proliferation, activation, and differentiation, including control of cell survival or death by apoptosis or cytotoxicity (Armitage, R.J., *Curr. Opin. Immunol.*, 6:407 (1994) and Smith, C.A., *Cell*, 75:959 (1994)).

TNF is produced by a number of cell types, including monocytes, fibroblasts, T cells, natural killer (NK) cells and predominately by activated macrophages. TNF- α has been reported to have a role in the rapid necrosis of tumors, immunostimulation, autoimmune disease, graft rejection, resistance to parasites, producing an anti-viral response, septic shock, growth regulation, vascular endothelium effects and metabolic effects. TNF- α also triggers endothelial cells to secrete various factors, including PAF-1, IL-1, GM-CSF and IL-6 to promote cell proliferation. In addition, TNF- α up-regulates various cell adhesion molecules such as E-Selectin, ICAM-1 and VCAM-1.

The first step in the induction of the various cellular responses mediated by the members of the TNF ligand superfamily is their binding to specific cell surface receptors. The TNF receptor superfamily contains at present ten known membrane proteins and several viral open reading frames encoding TNFR-related molecules. The p75 low-affinity Nerve Growth Factor (NGF) receptor was the first cloned receptor of this family (Johnson, D. et al. *Cell*, 47:545 (1986)). Subsequently, cloning of two specific receptors for TNF show that they were related to the NGF receptor (Loetscher, H. et al., *Cell*, 61:351 (1990)). In recent years, a new type I-transmembrane TNF receptor superfamily has been established. This family includes the p75 nerve growth factor receptor, p60 TNFR-I, p80 TNFR-II, TNFR-RP/TNFR-III, CD27, CD30, CD40, 4-1BB, OX40 and FAS/APO-1. In addition, several viral open reading frames encoding soluble TNF receptors have been identified, such as SFV-T2 in Shope fibroma virus (Smith, C.A. et al., *Biochem. Biophys. Res. Commun.*, 176:335, 1991) and Va53 or SaIF19R in vaccinia virus (Howard, S.T., *Virology*, 180:633, 1991). These receptors are characterized by multiple cysteine-rich domains in the extracellular (amino-terminal) domain, which have been shown to be involved in ligand binding. The average homology in the cysteine-rich extracellular region between the human family members are in the range of 25 to 30%.

Clearly, there is a need for factors that regulate activation, and differentiation of normal and abnormal cells. There is a need, therefore, for identification and characterization of such factors that modulate activation and differentiation of cells, both normally and in disease states. In particular, there is a need to isolate and characterize additional TNF ligands akin to members of the TNF ligand super-family that control apoptosis of transformed cell lines, mediate cell activation and proliferation and are functionally linked as primary mediators of immune regulation and inflammatory response, and, among other things, can play a role in preventing, ameliorating or correcting dysfunctions or diseases.

SUMMARY OF THE INVENTION

Toward these ends, and others, it is an object of the present invention to provide novel polypeptides, referred to as novel TNF delta and TNF epsilon which have been putatively identified as being tumor necrosis factor ligands by homology between the amino acid sequence set out in Figures 1 and 2 and known amino acid sequences of other proteins in the tumor necrosis factor family such as human TNF α and TNF β .

The polypeptides of the present invention have been identified as a novel members of the TNF ligand super-family based on structural and biological similarities.

It is a further object of the invention, moreover, to provide polynucleotides that encode TNF delta and TNF epsilon, particularly polynucleotides that encode the polypeptide herein designated TNF delta and TNF epsilon.

In a particularly preferred embodiment of this aspect of the invention the polynucleotides comprise the region encoding human TNF delta and TNF epsilon in the sequences set out in Figures 1 and 2.

In accordance with this aspect of the invention there are provided isolated nucleic acid molecules encoding human TNF delta, including mRNAs, cDNAs, genomic DNAs and, in further embodiments of this aspect of the invention, biologically, diagnostically, clinically or therapeutically useful variants, analogs or derivatives thereof, or fragments thereof, including fragments of the variants, analogs and derivatives.

Among the particularly preferred embodiments of this aspect of the invention are naturally occurring allelic variants of human TNF delta and TNF epsilon.

In accordance with this aspect of the present invention there are provided isolated nucleic acid molecules encoding a mature human TNF delta polypeptide expressed by

the human cDNA contained in ATCC Deposit No. 97377 deposited on December 8, 1995 and a mature human TNF epsilon polypeptide expressed by the human cDNA contained in ATCC Deposit No. 97457 deposited on March 1, 1996.

It also is an object of the invention to provide TNF delta polypeptides, particularly human TNF delta and TNF epsilon polypeptides, that destroy some transformed cell lines, mediate cell activation and proliferation and are functionally linked as primary mediators of immune regulation and inflammatory response.

In accordance with this aspect of the invention there are provided novel polypeptides of human origin referred to herein as TNF delta and TNF epsilon as well as biologically, diagnostically or therapeutically useful fragments, variants and derivatives thereof, variants and derivatives of the fragments, and analogs of the foregoing.

Among the particularly preferred embodiments of this aspect of the invention are variants of human TNF delta and TNF epsilon encoded by naturally occurring alleles of the human TNF delta and TNF epsilon gene.

It is another object of the invention to provide a process for producing the aforementioned polypeptides, polypeptide fragments, variants and derivatives, fragments of the variants and derivatives, and analogs of the foregoing. In a preferred embodiment of this aspect of the invention there are provided methods for producing the aforementioned TNF delta and TNF epsilon polypeptides comprising culturing host cells having expressibly incorporated therein an exogenously-derived human TNF delta-encoding polynucleotide and TNF epsilon-encoding polynucleotide under conditions for expression of human TNF delta and TNF epsilon in the host and then recovering the expressed polypeptide.

In accordance with another object the invention there are provided products, compositions, processes and methods that utilize the aforementioned polypeptides and polynucleotides for research, biological, clinical and therapeutic purposes, *inter alia*.

In accordance with certain preferred embodiments of this aspect of the invention, there are provided products, compositions and methods, *inter alia*, for, among other things: assessing TNF delta and TNF epsilon expression in cells by determining TNF delta and TNF epsilon polypeptides or TNF delta-encoding mRNA or TNF epsilon-encoding mRNA polypeptides; assaying genetic variation and aberrations, such as defects, in TNF delta and TNF epsilon genes; and administering a TNF delta or TNF epsilon polypeptide or polynucleotide to an organism to augment TNF delta or TNF epsilon function or remediate TNF delta or TNF epsilon dysfunction.

In accordance with certain preferred embodiments of this and other aspects of the invention there are provided polynucleotides and in particular probes that hybridize to human TNF delta or TNF epsilon sequences.

In certain additional preferred embodiments of this aspect of the invention there are provided antibodies against TNF delta or TNF epsilon polypeptides. In certain particularly preferred embodiments in this regard, the antibodies are highly selective for human TNF delta or TNF epsilon.

In accordance with another aspect of the present invention, there are provided TNF delta or TNF epsilon agonists. Among preferred agonists are molecules that mimic TNF delta or TNF epsilon, that bind to TNF delta-binding molecules or receptor molecules or to TNF epsilon-binding molecules or receptor molecules, and that elicit or augment TNF delta-induced or TNF epsilon-induced responses. Also among preferred agonists are molecules that interact with TNF delta and TNF epsilon or TNF delta and TNF epsilon polypeptides, or with other modulators of TNF delta activities,

and thereby potentiate or augment an effect of TNF delta and TNF epsilon or more than one effect of TNF delta and TNF epsilon.

In accordance with yet another aspect of the present invention, there are provided TNF delta and TNF epsilon antagonists. Among preferred antagonists are those which mimic TNF delta and TNF epsilon so as to bind to TNF delta and TNF epsilon receptors or binding molecules but not elicit a TNF delta- and TNF epsilon-induced response or more than one TNF delta- and TNF epsilon-induced response. Also among preferred antagonists are molecules that bind to or interact with TNF delta and TNF epsilon so as to inhibit an effect of TNF delta and TNF epsilon or more than one effect of TNF delta and TNF epsilon or which prevent expression of TNF delta and TNF epsilon.

The agonists and antagonists may be used to mimic, augment or inhibit the action of TNF delta and TNF epsilon polypeptides. They may be used, for instance, to prevent septic shock, inflammation, cerebral malaria, activation of the HIV virus, graft-host rejection, bone resorption, rheumatoid arthritis and cachexia.

In a further aspect of the invention there are provided compositions comprising a TNF delta and TNF epsilon polynucleotide or a TNF delta and TNF epsilon polypeptide for administration to cells in vitro, to cells ex vivo and to cells in vivo, or to a multicellular organism. In certain particularly preferred embodiments of this aspect of the invention, the compositions comprise a TNF delta and TNF epsilon polynucleotide for expression of a TNF delta and TNF epsilon polypeptide in a host organism for treatment of disease. Particularly preferred in this regard is expression in a human patient for treatment of a dysfunction associated with aberrant endogenous activity of TNF delta and TNF epsilon.

Other objects, features, advantages and aspects of the present invention will become apparent to those of skill from the following description. It should be

understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following description and from reading the other parts of the present disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

The following drawings depict certain embodiments of the invention. They are illustrative only and do not limit the invention otherwise disclosed herein.

Figure 1 shows the nucleotide and deduced amino acid sequence of human TNF delta.

Figure 2 shows the nucleotide and deduced amino acid sequence of human TNF epsilon.

Figure 3 shows the regions of similarity (alignment report) between amino acid sequences of TNF α , TNF β , TNF δ and TNF ϵ polypeptides.

Figure 4 shows structural and functional features of TNF delta deduced by the indicated techniques. as a function of amino acid sequence.

Figure 5 shows structural and functional features of TNF epsilon deduced by the indicated techniques. as a function of amino acid sequence.

The following illustrative explanations are provided to facilitate understanding of certain terms used frequently herein, particularly in the examples. The explanations are provided as a convenience and are not limitative of the invention.

The term "digestion" of DNA refers to catalytic cleavage of the DNA with a restriction enzyme that acts only at certain sequences in the DNA. The various restriction enzymes referred to herein are commercially available and their reaction conditions, cofactors and other requirements for use are known and routine to the skilled artisan.

For analytical purposes, typically, 1 μ g of plasmid or DNA fragment is digested with about 2 units of enzyme in about 20 μ l of reaction buffer. For the purpose of isolating DNA fragments for plasmid construction, typically 5 to 50 μ g of DNA are digested with 20 to 250 units of enzyme in proportionately larger volumes.

Appropriate buffers and substrate amounts for particular restriction enzymes are described in standard laboratory manuals, such as those referenced below, and they are specified by commercial suppliers.

Incubation times of about 1 hour at 37°C are ordinarily used, but conditions may vary in accordance with standard procedures, the supplier's instructions and the particulars of the reaction. After digestion, reactions may be analyzed, and fragments may be purified by electrophoresis through an agarose or polyacrylamide gel, using well known methods that are routine for those skilled in the art.

The term "genetic element" generally means a polynucleotide comprising a region that encodes a polypeptide or a region that regulates transcription or translation or other processes important to expression of the polypeptide in a host cell, or a polynucleotide comprising both a region that encodes a polypeptide and a region operably linked thereto that regulates expression.

Genetic elements may be comprised within a vector that replicates as an episomal element; that is, as a molecule physically independent of the host cell genome. They may be comprised within mini-chromosomes, such as those that arise during

amplification of transfected DNA by methotrexate selection in eukaryotic cells. Genetic elements also may be comprised within a host cell genome; not in their natural state but, rather, following manipulation such as isolation, cloning and introduction into a host cell in the form of purified DNA or in a vector, among others.

The term "isolated" means altered "by the hand of man" from its natural state; i.e., if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. For example, with respect to polynucleotides, the term isolated means that it is separated from the chromosome and cell in which it naturally occurs.

As part of or following isolation, such polynucleotides can be joined to other polynucleotides, for mutagenesis, to form fusion proteins, and for propagation or expression in a host, for instance. The isolated polynucleotides, alone or joined to other polynucleotides such as vectors, can be introduced into host cells, in culture or in whole organisms, after which such DNAs still would be isolated, as the term is used herein, because they would not be in their naturally occurring form or environment.

Similarly, the polynucleotides and polypeptides may occur in a composition, such as a media formulations, solutions for introduction of polynucleotides or polypeptides, for example, into cells, compositions or solutions for chemical or enzymatic reactions, for instance, which are not naturally occurring compositions, and, therein remain isolated polynucleotides or polypeptides within the meaning of that term as it is employed herein.

The term "ligation" refers to the process of forming phosphodiester bonds between two or more polynucleotides, which most often are double stranded DNAs.

Techniques for ligation are well known to the art and protocols for ligation are described in standard laboratory manuals and references, such as, for instance, Sambrook et al., *Molecular Cloning, a Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1989) and Maniatis et al., pg. 146, as cited below.

The term "oligonucleotide(s)" refers to relatively short polynucleotides. Often the term refers to single-stranded deoxyribonucleotides, but it can refer as well to single- or double-stranded ribonucleotides, RNA:DNA hybrids and double-stranded DNAs, among others.

Oligonucleotides, such as single-stranded DNA probe oligonucleotides, often are synthesized by chemical methods, such as those implemented on automated oligonucleotide synthesizers. However, oligonucleotides can be made by a variety of other methods, including in vitro recombinant DNA-mediated techniques and by expression of DNAs in cells and organisms.

Initially, chemically synthesized DNAs typically are obtained without a 5' phosphate. The 5' ends of such oligonucleotides are not substrates for phosphodiester bond formation by ligation reactions that employ DNA ligases typically used to form recombinant DNA molecules. Where ligation of such oligonucleotides is desired, a phosphate can be added by standard techniques, such as those that employ a kinase and ATP.

The 3' end of a chemically synthesized oligonucleotide generally has a free hydroxyl group and, in the presence of a ligase, such as T4 DNA ligase, readily will form a phosphodiester bond with a 5' phosphate of another polynucleotide, such as another oligonucleotide. As is well known, this reaction can be prevented selectively, where desired, by removing the 5' phosphates of the other polynucleotide(s) prior to ligation.

Plasmids generally are designated herein by a lower case p preceded and/or followed by capital letters and/or numbers, in accordance with standard naming conventions that are familiar to those of skill in the art. Starting plasmids disclosed herein are either commercially available, publicly available on an unrestricted basis, or can be constructed from available plasmids by routine application of well known, published procedures. Many plasmids and other cloning and expression vectors that can be used in accordance with the present invention are well known and readily available to those of skill in the art. Moreover, those of skill readily may construct any number of other plasmids suitable for use in the invention. The properties, construction and use of such plasmids, as well as other vectors, in the present invention will be readily apparent to those of skill from the present disclosure.

The term "polynucleotide(s)" generally refers to any polyribonucleotide or polydeoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. Thus, for instance, polynucleotides as used herein refers to, among others, single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, polynucleotide as used herein refers to triple-stranded regions comprising RNA or DNA or both RNA and DNA. The strands in such regions may be from the same molecule or from different molecules. The regions may include all of one or more of the molecules, but more typically involve only a region of some of the molecules. One of the molecules of a triple-helical region often is an oligonucleotide.

As used herein, the term polynucleotide includes DNAs or RNAs as described above that contain one or more modified bases. Thus, DNAs or RNAs with backbones modified for stability or for other reasons are "polynucleotides" as that term is intended herein. Moreover, DNAs or RNAs comprising unusual bases, such as inosine, or

modified bases, such as tritylated bases, to name just two examples, are polynucleotides as the term is used herein.

It will be appreciated that a great variety of modifications have been made to DNA and RNA that serve many useful purposes known to those of skill in the art. The term polynucleotide as it is employed herein embraces such chemically, enzymatically or metabolically modified forms of polynucleotides, as well as the chemical forms of DNA and RNA characteristic of viruses and cells, including simple and complex cells, *inter alia*.

The term "polypeptides," as used herein, includes all polypeptides as described below. The basic structure of polypeptides is well known and has been described in innumerable textbooks and other publications in the art. In this context, the term is used herein to refer to any peptide or protein comprising two or more amino acids joined to each other in a linear chain by peptide bonds. As used herein, the term refers to both short chains, which also commonly are referred to in the art as peptides, oligopeptides and oligomers, for example, and to longer chains, which generally are referred to in the art as proteins, of which there are many types.

It will be appreciated that polypeptides often contain amino acids other than the 20 amino acids commonly referred to as the 20 naturally occurring amino acids, and that many amino acids, including the terminal amino acids, may be modified in a given polypeptide, either by natural processes, such as processing and other post-translational modifications, but also by chemical modification techniques which are well known to the art. Even the common modifications that occur naturally in polypeptides are too numerous to list exhaustively here, but they are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature, and they are well known to those of skill in the art. Among the known modifications which may be present in polypeptides of the present invention are, to name an illustrative few, acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin,

covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of phosphatidylinositol, cross-linking, cyclization, disulfide bond formation, formation of covalent cross-links, formation of cystine, formation of pyroglutamate, formylation, gamma-carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodination, methylation, myristoylation, oxidation, proteolytic processing, phosphorylation, prenylation, racemization, selenoylation, sulfation, transfer-RNA mediated addition of amino acids to proteins such as arginylation, and ubiquitination.

Such modifications are well known to those of skill and have been described in great detail in the scientific literature. Several particularly common modifications, glycosylation, lipid attachment, sulfation, gamma-carboxylation of glutamic acid residues, hydroxylation and ADP-ribosylation, for instance, are described in most basic texts, such as, for instance *Proteins - Structure and Molecular Properties*, 2nd Ed., T. E. Creighton, W. H. Freeman and Company, New York (1993). Many detailed reviews are available on this subject, such as, for example, those provided by Wold, F., *Posttranslational Protein Modifications: Perspectives and Prospects*, pgs. 1-12 in *Posttranslational Covalent Modification of Proteins*, B. C. Johnson, Ed., Academic Press, New York (1983); Seifter *et al.*, *Analysis for protein modifications and nonprotein cofactors. Meth. Enzymol.*, 182: 626-646 (1990) and Rattan *et al.*, *Protein Synthesis: Posttranslational Modifications and Aging, Ann. N.Y. Acad. Sci.*, 663: 48-62 (1992).

It will be appreciated, as is well known and as noted above, that polypeptides are not always entirely linear. For instance, polypeptides may be branched as a result of ubiquitination, and they may be circular, with or without branching, generally as a result of posttranslation events, including natural processing event, and events brought about by human manipulation which do not occur naturally. Circular, branched and branched circular polypeptides may be synthesized by non-translational natural process and by entirely synthetic methods, as well.

Modifications can occur anywhere in a polypeptide, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. In fact, blockage of the amino or carboxyl group in a polypeptide, or both, by a covalent modification, is common in naturally occurring and synthetic polypeptides and such modifications may be present in polypeptides of the present invention, as well. For instance, the amino terminal residue of polypeptides made in *E. coli*, prior to proteolytic processing, almost invariably will be N-formylmethionine.

The modifications that occur in a polypeptide often will be a function of how it is made. For polypeptides made by expressing a cloned gene in a host, for instance, the nature and extent of the modifications in large part will be determined by the host cell posttranslational modification capacity and the modification signals present in the polypeptide amino acid sequence. For instance, as is well known, glycosylation often does not occur in bacterial hosts such as *E. coli*. Accordingly, when glycosylation is desired, a polypeptide should be expressed in a glycosylating host, generally a eukaryotic cell. Insect cells often carry out the same posttranslational glycosylations as mammalian cells and, for this reason, insect cell expression systems have been developed to express efficiently mammalian proteins having native patterns of glycosylation, *inter alia*. Similar considerations apply to other modifications. It will be appreciated that the same type of modification may be present in the same or varying degree at several sites in a given polypeptide. Also, a given polypeptide may contain many types of modifications. In general, as used herein, the term polypeptide encompasses all such modifications, particularly those that are present in polypeptides synthesized by expressing a polynucleotide in a host cell.

The term "variant(s)" of polynucleotides or polypeptides, as the term is used herein, are polynucleotides or polypeptides that differ from a reference polynucleotide or polypeptide, respectively. Variants in this sense are described below and elsewhere in the present disclosure in greater detail.

A polynucleotide variant is a polynucleotide that differs in nucleotide sequence from another, reference polynucleotide. Generally, differences are limited so that the nucleotide sequences of the reference and the variant are closely similar overall and, in many regions, identical. As noted below, changes in the nucleotide sequence of the variant may be silent. That is, they may not alter the amino acids encoded by the polynucleotide. Where alterations are limited to silent changes of this type a variant will encode a polypeptide with the same amino acid sequence as the reference. Also as noted below, changes in the nucleotide sequence of the variant may alter the amino acid sequence of a polypeptide encoded by the reference polynucleotide. Such nucleotide changes may result in amino acid substitutions, additions, deletions, fusions and truncations in the polypeptide encoded by the reference sequence, as discussed below.

A polypeptide variant is a polypeptide that differs in amino acid sequence from another, reference polypeptide. Generally, differences are limited so that the sequences of the reference and the variant are closely similar overall and, in many region, identical. A variant and reference polypeptide may differ in amino acid sequence by one or more substitutions, additions, deletions, fusions and truncations, which may be present in any combination.

The term "receptor molecule," as used herein, refers to molecules which bind or interact specifically with TNF delta or TNF epsilon polypeptides of the present invention, including not only classic receptors, which are preferred, but also other molecules that specifically bind to or interact with polypeptides of the invention (which also may be referred to as "binding molecules" and "interaction molecules," respectively and as "TNF delta binding molecules" and "TNF delta interaction molecules" or "TNF epsilon binding molecules" and "TNF epsilon interaction molecules." Binding between polypeptides of the invention and such molecules, including receptor or binding or interaction molecules may be exclusive to polypeptides of the invention, which is very highly preferred, or it may be highly specific for polypeptides of the invention, which is highly preferred, or it may be highly specific to a group of proteins that includes

polypeptides of the invention, which is preferred, or it may be specific to several groups of proteins at least one of which includes polypeptides of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to novel TNF delta and TNF epsilon polypeptides and polynucleotides, among other things, as described in greater detail below. In particular, the invention relates to polypeptides and polynucleotides which are related by amino acid sequence homology to the TNF ligand superfamily. The invention relates especially to TNF delta having the nucleotide and amino acid sequences set out in Figure 1, and to the TNF nucleotide and amino acid sequences of the human cDNA in ATCC Deposit No. 97377. The invention also relates especially to TNF epsilon having the nucleotide and amino acid sequences set out in Figure 2, and to the TNF epsilon nucleotide and amino acid sequences of the human cDNA in ATCC Deposit No. 97457. The deposits are hereinafter referred to as the deposited clones or as "the cDNA of the deposited clones." It will be appreciated that the nucleotide and amino acid sequences set out in Figures 1 and 2 were obtained by sequencing the human cDNA of the deposited clones. Hence, the sequence of the deposited clone is controlling as to any discrepancies between the two and any reference to the sequences of Figures 1 and 2 include reference to the sequences of the human cDNA's of the deposited clones.

In accordance with one aspect of the present invention, there are provided isolated polynucleotides which encode the TNF delta and TNF epsilon polypeptides having the deduced amino acid sequences of Figures 1 and 2.

Using the information provided herein, such as the polynucleotide sequence set out in Figure 1, a polynucleotide of the present invention encoding human TNF delta polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA from cells of human tissue as starting material. Illustrative of the invention, the polynucleotide set out in Figure 1 was discovered in a cDNA library derived from cells of human heart tissue.

Human TNF delta of the invention is structurally related to other proteins of the TNF ligand superfamily, as shown by the results of sequencing the cDNA encoding human TNF delta in the deposited clone. The cDNA sequence thus obtained is set out in Figure 1. It contains an open reading frame encoding a protein of about 233 amino acid residues with a deduced molecular weight of about 25.871 kDa. The protein exhibits greatest homology to TNF α , among known proteins. The entire amino acid sequence of TNF delta of Figure 1 has about 38% identity to the amino acid sequence of TNF α .

A polynucleotide of the present invention encoding human TNF epsilon polypeptide may be obtained using standard cloning and screening procedures, such as those for cloning cDNAs using mRNA from cells of human tissue as starting material. Illustrative of the invention, the polynucleotide set out in Figure 2 was discovered in a cDNA library derived from cells of human heart tissue.

Human TNF epsilon of the invention is structurally related to other proteins of the TNF ligand superfamily, as shown by the results of sequencing the cDNA encoding human TNF epsilon in the deposited clone. The cDNA sequence thus obtained is set out in Figure 2. The TNF epsilon sequence is nearly identical to the sequence of TNF delta as set out in Figure 1 minus the initial 50 amino acids and a region of TNF delta comprising amino acid 86 to amino acid 92. Accordingly, TNF epsilon is a splicing variant of TNF delta. TNF epsilon comprises 168 amino acid residues and the sequence of Figure 2 shows the mature protein of TNF epsilon without any N-terminal hydrophobic region. The protein exhibits greatest homology to TNF α . TNF epsilon of Figure 2 has about 20% identity to the amino acid sequence of TNF α .

Polynucleotides of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced by chemical synthetic techniques or by a combination thereof. The DNA may be double-stranded or single-stranded. Single-stranded DNA

may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

The coding sequence which encodes the polypeptide may be identical to the coding sequence of the polynucleotide shown in Figures 1 and 2. It also may be a polynucleotide with a different sequence, which, as a result of the redundancy (degeneracy) of the genetic code, encodes the polypeptide of the DNA of Figures 1 and 2.

Polynucleotides of the present invention which encode the polypeptide of Figures 1 and 2 may include, but are not limited to the coding sequence for the mature polypeptide, by itself; the coding sequence for the mature polypeptide and additional coding sequences, such as those encoding a leader or secretory sequence, such as a pre-, or pro- or prepro- protein sequence; the coding sequence of the mature polypeptide, with or without the aforementioned additional coding sequences, together with additional, non-coding sequences, including for example, but not limited to introns and non-coding 5' and 3' sequences, such as the transcribed, non-translated sequences that play a role in transcription, mRNA processing - including splicing and polyadenylation signals, for example - ribosome binding and stability of mRNA; additional coding sequence which codes for additional amino acids, such as those which provide additional functionalities.

Thus, for instance, the polypeptide may be fused to a marker sequence, such as a peptide, which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker sequence is a hexa-histidine peptide, such as the tag provided in the pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al.*, *Proc. Natl. Acad. Sci., USA*, 86:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the fusion protein. The HA tag corresponds to an epitope derived of

influenza hemagglutinin protein, which has been described by Wilson *et al.*, *Cell*, 37:767 (1984), for instance.

In accordance with the foregoing, the term "polynucleotide encoding a polypeptide" as used herein encompasses polynucleotides which include a sequence encoding a polypeptide of the present invention, particularly the human TNF delta and TNF epsilon having the amino acid sequences set out in Figures 1 and 2. The term encompasses polynucleotides that include a single continuous region or discontinuous regions encoding the polypeptide (for example, interrupted by introns) together with additional regions, that also may contain coding and/or non-coding sequences.

The present invention further relates to variants of the herein above described polynucleotides which encode for fragments, analogs and derivatives of the polypeptide having the deduced amino acid sequence of Figures 1 and 2. A variant of the polynucleotide may be a naturally occurring variant such as a naturally occurring allelic variant, or it may be a variant that is not known to occur naturally. Such non-naturally occurring variants of the polynucleotide may be made by mutagenesis techniques, including those applied to polynucleotides, cells or organisms.

Among variants in this regard are variants that differ from the aforementioned polynucleotides by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. The variants may be altered in coding or non-coding regions or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions.

Among the particularly preferred embodiments of the invention in this regard are polynucleotides encoding polypeptides having the amino acid sequence of TNF delta and TNF epsilon set out in Figures 1 and 2; variants, analogs, derivatives and fragments thereof, and fragments of the variants, analogs and derivatives.

Further particularly preferred in this regard are polynucleotides encoding TNF delta and TNF epsilon which have the amino acid sequence of the TNF delta and TNF epsilon polypeptide of Figures 1 and 2 in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNF delta and TNF epsilon. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polynucleotide encoding polypeptides having the amino acid sequence of Figures 1 and 2, without substitutions. Further preferred embodiments of the invention are polynucleotides that are at least 70% identical to a polynucleotide encoding the TNF delta and TNF epsilon polypeptide having the amino acid sequence set out in Figures 1 and 2, and polynucleotides which are complementary to such polynucleotides. Alternatively, most highly preferred are polynucleotides that comprise a region that is at least 80% identical to a polynucleotide encoding the TNF delta and TNF epsilon polypeptide and polynucleotides complementary thereto. In this regard, polynucleotides at least 90% identical to the same are particularly preferred, and among these particularly preferred polynucleotides, those with at least 95% are especially preferred. Furthermore, those with at least 97% are highly preferred among those with at least 95%, and among these those with at least 98% and at least 99% are particularly highly preferred, with at least 99% being the more preferred.

Particularly preferred embodiments in this respect, moreover, are polynucleotides which encode polypeptides which retain substantially the same biological function or activity as the mature polypeptide encoded by the cDNA of Figures 1 and 2.

The present invention further relates to polynucleotides that hybridize to the herein above-described sequences. In this regard, the present invention especially relates to polynucleotides which hybridize under stringent conditions to the herein above-described polynucleotides. As herein used, the term "stringent conditions" means

hybridization will occur when at least 95% and preferably at least 97% of the bases between sequences are complementary (e.g., G:C; A:T).

As discussed additionally herein regarding polynucleotide assays of the invention, for instance, polynucleotides of the invention as discussed above, may be used as a hybridization probe for cDNA and genomic DNA to isolate full-length cDNAs and genomic clones encoding TNF delta and TNF epsilon and to isolate cDNA and genomic clones of other genes that have a high sequence similarity to the human TNF delta and TNF epsilon gene. Such probes generally will comprise at least 15 bases. Preferably, such probes will have at least 30 bases and may have at least 50 bases.

For example, the coding region of the TNF delta and TNF epsilon gene may be isolated by screening using the known DNA sequence to synthesize an oligonucleotide probe. A labeled oligonucleotide having a sequence complementary to that of a gene of the present invention is then used to screen a library of human cDNA, genomic DNA or mRNA to determine which members of the library the probe hybridizes to.

The polynucleotides and polypeptides of the present invention may be employed as research reagents and materials for discovery of treatments and diagnostics to human disease, as further discussed herein relating to polynucleotide assays, *inter alia*.

The polynucleotides may encode a polypeptide which is the mature protein plus additional amino or carboxyl-terminal amino acids, or amino acids interior to the mature polypeptide (when the mature form has more than one polypeptide chain, for instance). Such sequences may play a role in processing of a protein from precursor to a mature form, may facilitate protein trafficking, may prolong or shorten protein half-life or may facilitate manipulation of a protein for assay or production, among other things. As generally is the case *in situ*, the additional amino acids may be processed away from the mature protein by cellular enzymes.

A precursor protein, having the mature form of the polypeptide fused to one or more prosequences may be an inactive form of the polypeptide. When prosequences are removed such inactive precursors generally are activated. Some or all of the prosequences may be removed before activation. Generally, such precursors are called proproteins.

In sum, a polynucleotide of the present invention may encode a mature protein, a mature protein plus a leader sequence (which may be referred to as a preprotein), a precursor of a mature protein having one or more prosequences which are not the leader sequences of a preprotein, or a preproprotein, which is a precursor to a proprotein, having a leader sequence and one or more prosequences, which generally are removed during processing steps that produce active and mature forms of the polypeptide.

Deposits containing human TNF delta and human TNF epsilon cDNA have been deposited with the American Type Culture Collection, as noted above. Also as noted above, the cDNA deposit is referred to herein as "the deposited clone" or as "the cDNA of the deposited clone." The clones were deposited with the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, USA, on December 8, 1995 and March 1, 1996, and assigned ATCC Deposit No. 97377 and 97457, respectively. The deposited materials are pBluescript SK (-) plasmids (Stratagene, La Jolla, CA) that contains the full length TNF delta and TNF epsilon human cDNA.

The deposits have been made under the terms of the Budapest Treaty on the international recognition of the deposit of micro-organisms for purposes of patent procedure. The strains will be irrevocably and without restriction or condition released to the public upon the issuance of a patent. The deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required for enablement, such as that required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited material, as well as the amino acid

sequence of the polypeptide encoded thereby, are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited materials, and no such license is hereby granted.

The present invention further relates to human TNF delta and TNF epsilon polypeptides having the deduced amino acid sequences of Figures 1 and 2. The polypeptide of the present invention may be a recombinant polypeptide, a natural polypeptide or a synthetic polypeptide. In certain preferred embodiments it is a recombinant polypeptide.

The invention also relates to fragments, analogs and derivatives of these polypeptides. The terms "fragment," "derivative" and "analog" when referring to the polypeptide of Figures 1 and 2 means a polypeptide which retains essentially the same biological function or activity as such polypeptide. Thus, an analog includes a proprotein which can be activated by cleavage of the proprotein portion to produce an active mature polypeptide.

The fragment, derivative or analog of the polypeptide of Figures 1 and 2 may be (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code, or (ii) one in which one or more of the amino acid residues includes a substituent group, or (iii) one in which the mature polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol), or (iv) one in which the additional amino acids are fused to the mature polypeptide, such as a leader or secretory sequence or a sequence which is employed for purification of the mature polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Among the particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of TNF delta and TNF epsilon set out in Figures 1 and 2, variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments. Alternatively, particularly preferred embodiments of the invention in this regard are polypeptides having the amino acid sequence of the TNF delta and TNF epsilon of the human cDNA in the deposited clone, variants, analogs, derivatives and fragments thereof, and variants, analogs and derivatives of the fragments.

Among preferred variants are those that vary from a reference by conservative amino acid substitutions. Such substitutions are those that substitute a given amino acid in a polypeptide by another amino acid of like characteristics. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Further particularly preferred in this regard are variants, analogs, derivatives and fragments, and variants, analogs and derivatives of the fragments, having the amino acid sequence of the TNF delta and TNF epsilon polypeptide of Figures 1 and 2 or of the cDNA in the deposited clone, in which several, a few, 5 to 10, 1 to 5, 1 to 3, 2, 1 or no amino acid residues are substituted, deleted or added, in any combination. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the TNF delta and TNF epsilon. Also especially preferred in this regard are conservative substitutions. Most highly preferred are polypeptides having the amino acid sequence of Figures 1 and 2 without substitutions.

The polypeptides and polynucleotides of the present invention are preferably provided in an isolated form, and preferably are purified to homogeneity.

The TGF delta polypeptides of the present invention include the polypeptide of SEQ ID NO:2 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:2 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:2 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:2 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

The TGF epsilon polypeptides of the present invention include the polypeptide of SEQ ID NO:4 (in particular the mature polypeptide) as well as polypeptides which have at least 70% similarity (preferably at least 70% identity) to the polypeptide of SEQ ID NO:4 and more preferably at least 90% similarity (more preferably at least 90% identity) to the polypeptide of SEQ ID NO:4 and still more preferably at least 95% similarity (still more preferably at least 95% identity) to the polypeptide of SEQ ID NO:4 and also include portions of such polypeptides with such portion of the polypeptide generally containing at least 30 amino acids and more preferably at least 50 amino acids.

As known in the art "similarity" between two polypeptides is determined by comparing the amino acid sequence and its conserved amino acid substitutes of one polypeptide to the sequence of a second polypeptide.

Fragments or portions of the polypeptides of the present invention may be employed for producing the corresponding full-length polypeptide by peptide synthesis; therefore, the fragments may be employed as intermediates for producing the full-length

polypeptides. Fragments or portions of the polynucleotides of the present invention may be used to synthesize full-length polynucleotides of the present invention.

A fragment is a polypeptide having an amino acid sequence that entirely is the same as part but not all of the amino acid sequence of the aforementioned TNF delta and TNF epsilon polypeptides and variants or derivatives thereof. Such fragments may be "free-standing," i.e., not part of or fused to other amino acids or polypeptides, or they may be comprised within a larger polypeptide of which they form a part or region. When comprised within a larger polypeptide, the presently discussed fragments most preferably form a single continuous region. However, several fragments may be comprised within a single larger polypeptide. For instance, certain preferred embodiments relate to a fragment of a TNF delta and TNF epsilon polypeptide of the present comprised within a precursor polypeptide designed for expression in a host and having heterologous pre and pro-polypeptide regions fused to the amino terminus of the TNF delta and TNF epsilon fragment and an additional region fused to the carboxyl terminus of the fragment. Therefore, fragments in one aspect of the meaning intended herein, refers to the portion or portions of a fusion polypeptide or fusion protein derived from TNF delta and TNF epsilon.

As representative examples of polypeptide fragments of the invention, there may be mentioned those which have from about 30 to about 233 amino acids. In this context, "about" includes the particularly recited range and ranges larger or smaller by several, a few, 5, 4, 3, 2 or 1 amino acid at either extreme or at both extremes. For instance, about 100 to 233 amino acids in this context means a polypeptide fragment of 100 plus or minus several, a few, 5, 4, 3, 2 or 1 amino acids to 233 plus or minus several a few, 5, 4, 3, 2 or 1 amino acid residues, i.e., ranges as broad as 100 minus several amino acids to 233 plus several amino acids to as narrow as 100 plus several amino acids to 233 minus several amino acids.

Highly preferred in this regard are the recited ranges plus or minus as many as 5 amino acids at either or at both extremes. Particularly highly preferred are the recited ranges plus or minus as many as 3 amino acids at either or at both the recited extremes. Especially particularly highly preferred are ranges plus or minus 1 amino acid at either or at both extremes or the recited ranges with no additions or deletions. Most highly preferred of all in this regard are fragments from about 15 to about 233 amino acids.

Among especially preferred fragments of the invention are truncation mutants of TNF delta and TNF epsilon. Truncation mutants include TNF delta and TNF epsilon polypeptides having the amino acid sequence of Figures 1 and 2, or of variants or derivatives thereof, except for deletion of a continuous series of residues (that is, a continuous region, part or portion) that includes the amino terminus, or a continuous series of residues that includes the carboxyl terminus or, as in double truncation mutants, deletion of two continuous series of residues, one including the amino terminus and one including the carboxyl terminus. Fragments having the size ranges set out about also are preferred embodiments of truncation fragments, which are especially preferred among fragments generally.

Also preferred in this aspect of the invention are fragments characterized by structural or functional attributes of TNF delta and TNF epsilon. Preferred embodiments of the invention in this regard include fragments that comprise alpha-helix and alpha-helix forming regions ("alpha-regions"), beta-sheet and beta-sheet-forming regions ("beta-regions"), turn and turn-forming regions ("turn-regions"), coil and coil-forming regions ("coil-regions"), hydrophilic regions, hydrophobic regions, alpha amphipathic regions, beta amphipathic regions, flexible regions, surface-forming regions and high antigenic index regions of TNF delta and TNF epsilon.

Certain preferred regions in these regards are set out in Figure 4 for TNF delta and Figure 5 for TNF epsilon, and include, but are not limited to, regions of the aforementioned types identified by analysis of the amino acid sequence set out in Figures

1 and 2. As set out in Figures 4 and 5, such preferred regions include Garnier-Robson alpha-regions, beta-regions, turn-regions and coil-regions, Chou-Fasman alpha-regions, beta-regions and turn-regions, Kyte-Doolittle hydrophilic regions and hydrophilic regions, Eisenberg alpha and beta amphipathic regions, Karplus-Schulz flexible regions, Emami surface-forming regions and Jameson-Wolf high antigenic index regions.

Among highly preferred fragments in this regard are those that comprise regions of TNF delta and TNF epsilon that combine several structural features, such as several of the features set out above. In this regard, the regions defined by the residues following the signal peptide region of Figures 1, 2, 4 and 5, which all are characterized by amino acid compositions highly characteristic of turn-regions, hydrophilic regions, flexible-regions, surface-forming regions, and high antigenic index-regions, are especially highly preferred regions. Such regions may be comprised within a larger polypeptide or may be by themselves a preferred fragment of the present invention, as discussed above. It will be appreciated that the term "about" as used in this paragraph has the meaning set out above regarding fragments in general.

Further preferred regions are those that mediate activities of TNF delta and TNF epsilon. Most highly preferred in this regard are fragments that have a chemical, biological or other activity of TNF delta and TNF epsilon, including those with a similar activity or an improved activity, or with a decreased undesirable activity. Highly preferred in this regard are fragments that contain regions that are homologs in sequence, or in position, or in both sequence and to active regions of related polypeptides, such as the related polypeptides set out in Figure 3, including human TNF α and β . Among particularly preferred fragments in these regards are truncation mutants, as discussed above.

It will be appreciated that the invention also relates to, among others, polynucleotides encoding the aforementioned fragments, polynucleotides that hybridize to polynucleotides encoding the fragments, particularly those that hybridize under

stringent conditions, and polynucleotides, such as PCR primers, for amplifying polynucleotides that encode the fragments. In these regards, preferred polynucleotides are those that correspondent to the preferred fragments, as discussed above.

The present invention also relates to vectors which include polynucleotides of the present invention, host cells which are genetically engineered with vectors of the invention and the production of polypeptides of the invention by recombinant techniques.

Host cells can be genetically engineered to incorporate polynucleotides and express polypeptides of the present invention. For instance, polynucleotides may be introduced into host cells using well known techniques of infection, transduction, transfection, transvection and transformation. The polynucleotides may be introduced alone or with other polynucleotides. Such other polynucleotides may be introduced independently, co-introduced or introduced joined to the polynucleotides of the invention. Thus, for instance, polynucleotides of the invention may be transfected into host cells with another, separate, polynucleotide encoding a selectable marker, using standard techniques for co-transfection and selection in, for instance, mammalian cells. In this case the polynucleotides generally will be stably incorporated into the host cell genome.

Alternatively, the polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. The vector construct may be introduced into host cells by the aforementioned techniques. Generally, a plasmid vector is introduced as DNA in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. Electroporation also may be used to introduce polynucleotides into a host. If the vector is a virus, it may be packaged in vitro or introduced into a packaging cell and the packaged virus may be transduced into cells. A wide variety of techniques suitable for making polynucleotides and for introducing polynucleotides into cells in accordance with this aspect of the invention are well known and routine to those of skill in the art. Such techniques are reviewed at length in

Sambrook *et al.* cited above, which is illustrative of the many laboratory manuals that detail these techniques. In accordance with this aspect of the invention the vector may be, for example, a plasmid vector, a single or double-stranded phage vector, a single or double-stranded RNA or DNA viral vector. Such vectors may be introduced into cells as polynucleotides, preferably DNA, by well known techniques for introducing DNA and RNA into cells. The vectors, in the case of phage and viral vectors also may be and preferably are introduced into cells as packaged or encapsidated virus by well known techniques for infection and transduction. Viral vectors may be replication competent or replication defective. In the latter case viral propagation generally will occur only in complementing host cells.

Preferred among vectors, in certain respects, are those for expression of polynucleotides and polypeptides of the present invention. Generally, such vectors comprise cis-acting control regions effective for expression in a host operatively linked to the polynucleotide to be expressed. Appropriate trans-acting factors either are supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression. Such specific expression may be inducible expression or expression only in certain types of cells or both inducible and cell-specific. Particularly preferred among inducible vectors are vectors that can be induced for expression by environmental factors that are easy to manipulate, such as temperature and nutrient additives. A variety of vectors suitable to this aspect of the invention, including constitutive and inducible expression vectors for use in prokaryotic and eukaryotic hosts, are well known and employed routinely by those of skill in the art.

The engineered host cells can be cultured in conventional nutrient media, which may be modified as appropriate for, *inter alia*, activating promoters, selecting transformants or amplifying genes. Culture conditions, such as temperature, pH and the

like, previously used with the host cell selected for expression generally will be suitable for expression of polypeptides of the present invention as will be apparent to those of skill in the art.

A great variety of expression vectors can be used to express a polypeptide of the invention. Such vectors include chromosomal, episomal and virus-derived vectors *e.g.*, vectors derived from bacterial plasmids, from bacteriophage, from yeast episomes, from yeast chromosomal elements, from viruses such as baculoviruses, papova viruses, such as SV40, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as those derived from plasmid and bacteriophage genetic elements, such as cosmids and phagemids, all may be used for expression in accordance with this aspect of the present invention. Generally, any vector suitable to maintain, propagate or express polynucleotides to express a polypeptide in a host may be used for expression in this regard.

The appropriate DNA sequence may be inserted into the vector by any of a variety of well-known and routine techniques. In general, a DNA sequence for expression is joined to an expression vector by cleaving the DNA sequence and the expression vector with one or more restriction endonucleases and then joining the restriction fragments together using T4 DNA ligase. Procedures for restriction and ligation that can be used to this end are well known and routine to those of skill. Suitable procedures in this regard, and for constructing expression vectors using alternative techniques, which also are well known and routine to those skill, are set forth in great detail in Sambrook et al. cited elsewhere herein.

The DNA sequence in the expression vector is operatively linked to appropriate expression control sequence(s), including, for instance, a promoter to direct mRNA transcription. Representatives of such promoters include the phage lambda PL promoter, the *E. coli* lac, trp and tac promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name just a few of the well-known promoters. It will

be understood that numerous promoters not mentioned are suitable for use in this aspect of the invention are well known and readily may be employed by those of skill in the manner illustrated by the discussion and the examples herein.

In general, expression constructs will contain sites for transcription initiation and termination, and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will include a translation initiating AUG at the beginning and a termination codon appropriately positioned at the end of the polypeptide to be translated.

In addition, the constructs may contain control regions that regulate as well as engender expression. Generally, in accordance with many commonly practiced procedures, such regions will operate by controlling transcription, such as repressor binding sites and enhancers, among others.

Vectors for propagation and expression generally will include selectable markers. Such markers also may be suitable for amplification or the vectors may contain additional markers for this purpose. In this regard, the expression vectors preferably contain one or more selectable marker genes to provide a phenotypic trait for selection of transformed host cells. Preferred markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture, and tetracycline or ampicillin resistance genes for culturing *E. coli* and other bacteria.

The vector containing the appropriate DNA sequence as described elsewhere herein, as well as an appropriate promoter, and other appropriate control sequences, may be introduced into an appropriate host using a variety of well known techniques suitable to expression therein of a desired polypeptide. Representative examples of appropriate hosts include bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and Bowes melanoma cells;

and plant cells. Hosts for of a great variety of expression constructs are well known, and those of skill will be enabled by the present disclosure readily to select a host for expressing a polypeptides in accordance with this aspect of the present invention.

More particularly, the present invention also includes recombinant constructs, such as expression constructs, comprising one or more of the sequences described above. The constructs comprise a vector, such as a plasmid or viral vector, into which such a sequence of the invention has been inserted. The sequence may be inserted in a forward or reverse orientation. In certain preferred embodiments in this regard, the construct further comprises regulatory sequences, including, for example, a promoter, operably linked to the sequence. Large numbers of suitable vectors and promoters are known to those of skill in the art, and there are many commercially available vectors suitable for use in the present invention.

The following vectors, which are commercially available, are provided by way of example. Among vectors preferred for use in bacteria are pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A, available from Stratagene; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. These vectors are listed solely by way of illustration of the many commercially available and well known vectors that are available to those of skill in the art for use in accordance with this aspect of the present invention. It will be appreciated that any other plasmid or vector suitable for, for example, introduction, maintenance, propagation or expression of a polynucleotide or polypeptide of the invention in a host may be used in this aspect of the invention.

Promoter regions can be selected from any desired gene using vectors that contain a reporter transcription unit lacking a promoter region, such as a

chloramphenicol acetyl transferase ("cat") transcription unit, downstream of restriction site or sites for introducing a candidate promoter fragment; i.e., a fragment that may contain a promoter. As is well known, introduction into the vector of a promoter-containing fragment at the restriction site upstream of the cat gene engenders production of CAT activity, which can be detected by standard CAT assays. Vectors suitable to this end are well known and readily available. Two such vectors are pKK232-8 and pCM7. Thus, promoters for expression of polynucleotides of the present invention include not only well known and readily available promoters, but also promoters that readily may be obtained by the foregoing technique, using a reporter gene.

Among known bacterial promoters suitable for expression of polynucleotides and polypeptides in accordance with the present invention are the *E. coli* lacI and lacZ and promoters, the T3 and T7 promoters, the gpt promoter, the lambda PR, PL promoters and the trp promoter. Among known eukaryotic promoters suitable in this regard are the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus ("RSV"), and metallothionein promoters, such as the mouse metallothionein-I promoter. Selection of appropriate vectors and promoters for expression in a host cell is a well known procedure and the requisite techniques for expression vector construction, introduction of the vector into the host and expression in the host are routine skills in the art.

The present invention also relates to host cells containing the above-described constructs discussed above. The host cell can be a higher eukaryotic cell, such as a mammalian cell, or a lower eukaryotic cell, such as a yeast cell, or the host cell can be a prokaryotic cell, such as a bacterial cell.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods

are described in many standard laboratory manuals, such as Davis *et al.* Basic Methods in Molecular Biology, (1986). Constructs in host cells can be used in a conventional manner to produce the gene product encoded by the recombinant sequence. Alternatively, the polypeptides of the invention can be synthetically produced by conventional peptide synthesizers.

Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989).

Generally, recombinant expression vectors will include origins of replication, a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence, and a selectable marker to permit isolation of vector containing cells after exposure to the vector. Among suitable promoters are those derived from the genes that encode glycolytic enzymes such as 3-phosphoglycerate kinase ("PGK"), a-factor, acid phosphatase, and heat shock proteins, among others. Selectable markers include the ampicillin resistance gene of *E. coli* and the *trp1* gene of *S. cerevisiae*.

Transcription of the DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

Polynucleotides of the invention, encoding the heterologous structural sequence of a polypeptide of the invention generally will be inserted into the vector using standard techniques so that it is operably linked to the promoter for expression. The polynucleotide will be positioned so that the transcription start site is located appropriately 5' to a ribosome binding site. The ribosome binding site will be 5' to the AUG that initiates translation of the polypeptide to be expressed. Generally, there will be no other open reading frames that begin with an initiation codon, usually AUG, and lie between the ribosome binding site and the initiating AUG. Also, generally, there will be a translation stop codon at the end of the polypeptide and there will be a polyadenylation signal and a transcription termination signal appropriately disposed at the 3' end of the transcribed region.

For secretion of the translated protein into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals but also additional heterologous functional regions. Thus, for instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification or during subsequent handling and storage. Also, region also may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art.

Suitable prokaryotic hosts for propagation, maintenance or expression of polynucleotides and polypeptides in accordance with the invention include *Escherichia*

coli, *Bacillus subtilis* and *Salmonella typhimurium*. Various species of *Pseudomonas*, *Streptomyces*, and *Staphylococcus* are suitable hosts in this regard. Moreover, many other hosts also known to those of skill may be employed in this regard.

As a representative but non-limiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, where the selected promoter is inducible it is induced by appropriate means (*e.g.*, temperature shift or exposure to chemical inducer) and cells are cultured for an additional period. Cells typically then are harvested by centrifugation, disrupted by physical or chemical means, and the resulting crude extract retained for further purification. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents, such methods are well known to those skilled in the art.

Various mammalian cell culture systems can be employed for expression, as well. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblast, described in Gluzman *et al.*, *Cell*, 23:175 (1981). Other cell lines capable of expressing a compatible vector include for example, the C127, 3T3, CHO, HeLa, human kidney 293 and BHK cell lines.

Mammalian expression vectors will comprise an origin of replication, a suitable promoter and enhancer, and also any necessary ribosome binding sites, polyadenylation

sites, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking non-transcribed sequences that are necessary for expression. In certain preferred embodiments in this regard DNA sequences derived from the SV40 splice sites, and the SV40 polyadenylation sites are used for required non-transcribed genetic elements of these types.

The polypeptides of the present invention can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography and lectin chromatography. Most preferably, high performance liquid chromatography ("HPLC") is employed for purification. Well known techniques for refolding protein may be employed to regenerate active conformation when the polypeptide is denatured during isolation and or purification.

Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells. Depending upon the host employed in a recombinant production procedure, the polypeptides of the present invention may be glycosylated or may be non-glycosylated. In addition, polypeptides of the invention may also include an initial modified methionine residue, in some cases as a result of host-mediated processes.

The polynucleotides and polypeptides of the present invention may be used in accordance with the present invention for a variety of applications, particularly those that make use of the chemical and biological properties TNF delta and TNF epsilon. Among these are applications in apoptosis of transformed cell lines, mediation of cell activation and proliferation and primary mediators of immune regulation antimicrobial,

antiviral and inflammatory response susceptibility to pathogens. Additional applications relate to diagnosis and to treatment of disorders of cells, tissues and organisms. These aspects of the invention are illustrated further by the following discussion.

This invention is also related to the use of the polynucleotides of the present invention to detect complementary polynucleotides such as, for example, as a diagnostic reagent. Detection of a mutated form of a polypeptide of the present invention associated with a dysfunction will provide a diagnostic tool that can add or define a diagnosis of a disease or susceptibility to a disease which results from under-expression over-expression or altered expression of polypeptide of the present invention, such as, for example, neoplasia such as tumors.

Individuals carrying mutations in a gene of the present invention may be detected at the DNA level by a variety of techniques. Nucleic acids for diagnosis may be obtained from a patient's cells, such as from blood, urine, saliva, tissue biopsy and autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR prior to analysis. PCR (Saiki *et al.*, *Nature*, 324: 163-166 1986). RNA or cDNA may also be used in the same ways. As an example, PCR primers complementary to the nucleic acid encoding TNF delta or TNF epsilon can be used to identify and analyze TNF delta or TNF epsilon expression and mutations. For example, deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Point mutations can be identified by hybridizing amplified DNA to radiolabeled TNF delta or TNF epsilon RNA or alternatively, radiolabeled TNF delta or TNF epsilon antisense DNA sequences. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase A digestion or by differences in melting temperatures.

Sequence differences between a reference gene and genes having mutations also may be revealed by direct DNA sequencing. In addition, cloned DNA segments may be employed as probes to detect specific DNA segments. The sensitivity of such

methods can be greatly enhanced by appropriate use of PCR or another amplification method. For example, a sequencing primer is used with double-stranded PCR product or a single-stranded template molecule generated by a modified PCR. The sequence determination is performed by conventional procedures with radiolabeled nucleotide or by automatic sequencing procedures with fluorescent-tags.

Genetic testing based on DNA sequence differences may be achieved by detection of alteration in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents. Small sequence deletions and insertions can be visualized by high resolution gel electrophoresis. DNA fragments of different sequences may be distinguished on denaturing formamide gradient gels in which the mobilities of different DNA fragments are retarded in the gel at different positions according to their specific melting or partial melting temperatures (see, e.g., Myers *et al.*, *Science*, 230:1242 1985).

Sequence changes at specific locations also may be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (e.g., Cotton *et al.*, *Proc. Natl. Acad. Sci., USA*, 85:4397-4401, 1985). Thus, the detection of a specific DNA sequence may be achieved by methods such as hybridization, RNase protection, chemical cleavage, direct DNA sequencing or the use of restriction enzymes, (e.g., restriction fragment length polymorphisms ("RFLP") and Southern blotting of genomic DNA. In addition to more conventional gel-electrophoresis and DNA sequencing, mutations also can be detected by *in situ* analysis.

The sequences of the present invention are also valuable for chromosome identification. The sequence is specifically targeted to and can hybridize with a particular location on an individual human chromosome. Moreover, there is a current need for identifying particular sites on the chromosome. Few chromosome marking reagents based on actual sequence data (repeat polymorphisms) are presently available for marking chromosomal location. The mapping of DNAs to chromosomes according

to the present invention is an important first step in correlating those sequences with genes associated with disease.

In certain preferred embodiments in this regard, the cDNA herein disclosed is used to clone genomic DNA of a gene of the present invention. This can be accomplished using a variety of well known techniques and libraries, which generally are available commercially. The genomic DNA the is used for *in situ* chromosome mapping using well known techniques for this purpose. Typically, in accordance with routine procedures for chromosome mapping, some trial and error may be necessary to identify a genomic probe that gives a good *in situ* hybridization signal.

In some cases, in addition, sequences can be mapped to chromosomes by preparing PCR primers (preferably 15-25 bp) from the cDNA. Computer analysis of the 3' untranslated region of the gene is used to rapidly select primers that do not span more than one exon in the genomic DNA, thus complicating the amplification process. These primers are then used for PCR screening of somatic cell hybrids containing individual human chromosomes. Only those hybrids containing the human gene corresponding to the primer will yield an amplified fragment.

PCR mapping of somatic cell hybrids is a rapid procedure for assigning a particular DNA to a particular chromosome. Using the present invention with the same oligonucleotide primers, sublocalization can be achieved with panels of fragments from specific chromosomes or pools of large genomic clones in an analogous manner. Other mapping strategies that can similarly be used to map to its chromosome include *in situ* hybridization, prescreening with labeled flow-sorted chromosomes and preselection by hybridization to construct chromosome specific-cDNA libraries.

Fluorescence *in situ* hybridization ("FISH") of a cDNA clone to a metaphase chromosomal spread can be used to provide a precise chromosomal location in one step. This technique can be used with cDNA as short as 50 or 60. For a review of this

technique, see Verma *et al.*, Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York (1988).

Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found, for example, in V. McKusick, Mendelian Inheritance in Man, available on line through Johns Hopkins University, Welch Medical Library. The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes).

Next, it is necessary to determine the differences in the cDNA or genomic sequence between affected and unaffected individuals. If a mutation is observed in some or all of the affected individuals but not in any normal individuals, then the mutation is likely to be the causative agent of the disease.

With current resolution of physical mapping and genetic mapping techniques, a cDNA precisely localized to a chromosomal region associated with the disease could be one of between 50 and 500 potential causative genes. (This assumes 1 megabase mapping resolution and one gene per 20 kb).

The present invention also relates to a diagnostic assays such as quantitative and diagnostic assays for detecting levels of a protein in the present invention in cells and tissues, including determination of normal and abnormal levels. Thus, for instance, a diagnostic assay in accordance with the invention for detecting over-expression of TNF protein of the present invention compared to normal control tissue samples may be used to detect the presence of neoplasia, for example. Assay techniques that can be used to determine levels of a protein, such as a protein of the present invention, in a sample derived from a host are well-known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-binding assays, Western Blot analysis and

ELISA assays. Among these ELISAs frequently are preferred. An ELISA assay initially comprises preparing an antibody specific to a protein of the present invention, preferably a monoclonal antibody. In addition a reporter antibody generally is prepared which binds to the monoclonal antibody. The reporter antibody is attached to a detectable reagent such as radioactive, fluorescent or enzymatic, which in this example is horseradish peroxidase enzyme.

To carry out an ELISA assay a sample is removed from a host and incubated on a solid support, *e.g.* a polystyrene dish, that binds the proteins in the sample. Any free protein binding sites on the dish are then covered by incubating with a non-specific protein such as bovine serum albumin. Next, the monoclonal antibody is incubated in the dish during which time the monoclonal antibodies attach to any protein of the present invention attached to the polystyrene dish. Unbound monoclonal antibody is washed out with buffer. The reporter antibody linked to horseradish peroxidase is placed in the dish resulting in binding of the reporter antibody to any monoclonal antibody bound to a protein of the present invention. Unattached reporter antibody is then washed out. Reagents for peroxidase activity, including a colorimetric substrate are then added to the dish. Immobilized peroxidase, linked to protein of the present invention through the primary and secondary antibodies, produces a colored reaction product. The amount of color developed in a given time period indicates the amount of protein of the present invention present in the sample. Quantitative results typically are obtained by reference to a standard curve.

A competition assay may be employed wherein antibodies specific to protein of the present invention attached to a solid support and labeled protein of the present invention and a sample derived from the host are passed over the solid support and the amount of label detected attached to the solid support can be correlated to a quantity of protein of the present invention in the sample.

The polypeptides, their fragments or other derivatives, or analogs thereof, or cells expressing them can be used as an immunogen to produce antibodies thereto. These antibodies can be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain, and humanized antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures known in the art may be used for the production of such antibodies and fragments.

Antibodies generated against the polypeptides corresponding to a sequence of the present invention can be obtained by direct injection of the polypeptides into an animal or by administering the polypeptides to an animal, preferably a nonhuman. The antibody so obtained will then bind the polypeptides itself. In this manner, even a sequence encoding only a fragment of the polypeptides can be used to generate antibodies binding the whole native polypeptides. Such antibodies can then be used to isolate the polypeptide from tissue expressing that polypeptide.

For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures can be used. Examples include the hybridoma technique (Kohler, G. and Milstein, C., *Nature*, 256:495-497 (1975), the trioma technique, the human B-cell hybridoma technique (Kozbor *et al.*, *Immunology Today*, 4:72 (1983) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole *et al.*, pg. 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985).

Techniques described for the production of single chain antibodies (U.S. Patent No. 4,946,778) can be adapted to produce single chain antibodies to immunogenic polypeptide products of this invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express humanized antibodies to immunogenic polypeptide products of this invention.

The above-described antibodies may be employed to isolate or to identify clones expressing the polypeptide or purify the polypeptide of the present invention by attachment of the antibody to a solid support for isolation and/or purification by affinity chromatography.

Thus, the polypeptides of the present invention of the present invention may be employed to inhibit neoplasia, such as tumor cell growth. The polypeptides of the present invention may be responsible for tumor destruction through apoptosis and cytotoxicity to certain cells. The polypeptides of the present invention also induce up-regulation of adhesion cells, for example, LFA-1, therefore, may be employed for wound-healing. The polypeptides of the present invention may also be employed to treat diseases which require growth promotion activity, for example, restenosis, since the polypeptides of the present invention have proliferation effects on cells of endothelial origin. The polypeptides of the present invention may, therefore, also be employed to regulate hematopoiesis in endothelial cell development.

The polypeptides of the present invention also stimulate the activation of T-cells, and may, therefore, be employed to stimulate an immune response against a variety of parasitic, bacterial and viral infections. The polypeptides of the present invention may also be employed in this respect to eliminate autoreactive T-cells to treat and/or prevent autoimmune diseases. An example of an autoimmune disease is Type I diabetes.

This invention also provides a method for identification of molecules, such as receptor molecules, that bind the proteins of the present invention. Genes encoding proteins that bind the proteins of the present invention, such as receptor proteins, can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. Such methods are described in many laboratory manuals such as, for instance, Coligan *et al.*, Current Protocols in Immunology 1(2): Chapter 5 (1991).

For instance, expression cloning may be employed for this purpose. To this end polyadenylated RNA is prepared from a cell responsive to the proteins of the present invention, a cDNA library is created from this RNA, the library is divided into pools and the pools are transfected individually into cells that are not responsive to the proteins of the present invention. The transfected cells then are exposed to labeled the proteins of the present invention. The proteins of the present invention can be labeled by a variety of well-known techniques including standard methods of radio-iodination or inclusion of a recognition site for a site-specific protein kinase. Following exposure, the cells are fixed and binding of cytostratin is determined. These procedures conveniently are carried out on glass slides.

Pools are identified of cDNA that produced TNF delta or TNF epsilon binding cells. Sub-pools are prepared from these positives, transfected into host cells and screened as described above. Using an iterative sub-pooling and re-screening process, one or more single clones that encode the putative binding molecule, such as a receptor molecule, can be isolated.

Alternatively a labeled ligand can be photoaffinity linked to a cell extract, such as a membrane or a membrane extract, prepared from cells that express a molecule that it binds, such as a receptor molecule. Cross-linked material is resolved by polyacrylamide gel electrophoresis ("PAGE") and exposed to X-ray film. The labeled complex containing the ligand-receptor can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing can be used to design unique or degenerate oligonucleotide probes to screen cDNA libraries to identify genes encoding the putative receptor molecule.

Polypeptides of the invention also can be used to assess TNF delta or TNF epsilon binding capacity of TNF delta or TNF epsilon binding molecules, such as receptor molecules, in cells or in cell-free preparations.

The invention also provides a method of screening compounds to identify those which enhance or block the action of TNF delta or TNF epsilon on cells, such as its interaction with TNF delta or TNF epsilon binding molecules such as receptor molecules. An agonist is a compound which increases the natural biological functions of polypeptides of the present invention or which functions in a manner similar to polypeptides of the present invention, while antagonists decrease or eliminate such functions.

For example, a cellular compartment, such as a membrane or a preparation thereof, such as a membrane-preparation, may be prepared from a cell that expresses a molecule that binds TNF delta or TNF epsilon, such as a molecule of a signaling or regulatory pathway modulated by TNF delta or TNF epsilon. The preparation is incubated with labeled TNF delta or TNF epsilon in the absence or the presence of a candidate molecule which may be a TNF delta or TNF epsilon agonist or antagonist. The ability of the candidate molecule to bind the binding molecule is reflected in decreased binding of the labeled ligand. Molecules which bind gratuitously, i.e., without inducing the effects of TNF delta or TNF epsilon on binding the TNF delta or TNF epsilon binding molecule, are most likely to be good antagonists. Molecules that bind well and elicit effects that are the same as or closely related to TNF delta or TNF epsilon are agonists.

TNF delta or TNF epsilon-like effects of potential agonists and antagonists may be measured, for instance, by determining activity of a second messenger system following interaction of the candidate molecule with a cell or appropriate cell preparation, and comparing the effect with that of TNF delta or TNF epsilon or molecules that elicit the same effects as TNF delta or TNF epsilon. Second messenger systems that may be useful in this regard include but are not limited to AMP guanylate cyclase, ion channel or phosphoinositide hydrolysis second messenger systems.

Another example of an assay for TNF delta or TNF epsilon antagonists is a competitive assay that combines TNF delta or TNF epsilon and a potential antagonist with membrane-bound TNF delta or TNF epsilon receptor molecules or recombinant TNF delta or TNF epsilon receptor molecules under appropriate conditions for a competitive inhibition assay. TNF delta or TNF epsilon can be labeled, such as by radioactivity, such that the number of TNF delta or TNF epsilon molecules bound to a receptor molecule can be determined accurately to assess the effectiveness of the potential antagonist.

Potential antagonists include small organic molecules, peptides, polypeptides and antibodies that bind to a polypeptide of the invention and thereby inhibit or extinguish its activity. Potential antagonists also may be small organic molecules, a peptide, a polypeptide such as a closely related protein or antibody that binds the same sites on a binding molecule, such as a receptor molecule, without inducing TNF delta or TNF epsilon-induced activities, thereby preventing the action of a polypeptide of the present invention by excluding it from binding to its receptor.

Another potential antagonist is a soluble form of the TNF delta or TNF epsilon receptor which binds to TNF delta or TNF epsilon and prevents it from interacting with membrane-bound TNF delta or TNF epsilon receptors. In this way, the receptors are not stimulated by their ligand.

Potential antagonists include a small molecule which binds to and occupies the binding site of the polypeptide thereby preventing binding to cellular binding molecules, such as receptor molecules, such that normal biological activity is prevented. Examples of small molecules include but are not limited to small organic molecules, peptides or non-peptide antagonists.

Other potential antagonists include antisense molecules. Antisense technology can be used to control gene expression through antisense DNA or RNA or through

triple-helix formation. Antisense techniques are discussed, for example, in Okano, J. *Neurochem.*, 56:560, 1991; Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988). Triple helix formation is discussed in, for instance Lee *et al.*, *Nucleic Acids Research*, 6:3073 (1979); Cooney *et al.*, *Science*, 241:456 (1988); and Dervan *et al.*, *Science*, 251:1360 (1991). The methods are based on binding of a polynucleotide to a complementary DNA or RNA. For example, the 5' coding portion of a polynucleotide that encodes the mature polypeptide of the present invention may be used to design an antisense RNA oligonucleotide of from about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription thereby preventing transcription and the production of TNF delta or TNF epsilon. The antisense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecule into TNF delta or TNF epsilon polypeptide. The oligonucleotides described above can also be delivered to cells such that the antisense RNA or DNA may be expressed in vivo to inhibit production of a polypeptide of the present invention.

The antagonists may be employed in a composition with a pharmaceutically acceptable carrier, *e.g.*, as hereinafter described.

The antagonists may be employed for instance to treat cachexia which is a lipid clearing defect resulting from a systemic deficiency of lipoprotein lipase, which is suppressed by TNF delta or TNF epsilon. The antagonists may also be employed to treat cerebral malaria in which polypeptides of the present invention appear to play a pathogenic role. The antagonists may also be employed to treat rheumatoid arthritis by inhibiting TNF delta or TNF epsilon induced production of inflammatory cytokines, such as IL1 in the synovial cells. When treating arthritis, the polypeptides of the present invention are preferably injected intra-articularly.

The antagonists may also be employed to prevent graft-host rejection by preventing the stimulation of the immune system in the presence of a graft.

The antagonists may also be employed to inhibit bone resorption and, therefore, to treat and/or prevent osteoporosis.

The antagonists may also be employed as anti-inflammatory agents, and to treat endotoxic shock. This critical condition results from an exaggerated response to bacterial and other types of infection.

The invention also relates to compositions comprising the polynucleotide or the polypeptides discussed above or the agonists or antagonists. Thus, the polypeptides of the present invention may be employed in combination with a non-sterile or sterile carrier or carriers for use with cells, tissues or organisms, such as a pharmaceutical carrier suitable for administration to a subject. Such compositions comprise, for instance, a media additive or a therapeutically effective amount of a polypeptide of the invention and a pharmaceutically acceptable carrier or excipient. Such carriers may include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol and combinations thereof. The formulation should suit the mode of administration.

The invention further relates to pharmaceutical packs and kits comprising one or more containers filled with one or more of the ingredients of the aforementioned compositions of the invention. Associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, reflecting approval by the agency of the manufacture, use or sale of the product for human administration.

Polypeptides and other compounds of the present invention may be employed alone or in conjunction with other compounds, such as therapeutic compounds.

The pharmaceutical compositions may be administered in any effective, convenient manner including, for instance, administration by topical, oral, anal, vaginal,

intravenous, intraperitoneal, intramuscular, subcutaneous, intranasal or intradermal routes among others.

The pharmaceutical compositions generally are administered in an amount effective for treatment or prophylaxis of a specific indication or indications. In general, the compositions are administered in an amount of at least about 10 $\mu\text{g/kg}$ body weight. In most cases they will be administered in an amount not in excess of about 8 mg/kg body weight per day. Preferably, in most cases, dose is from about 10 $\mu\text{g/kg}$ to about 1 mg/kg body weight, daily. It will be appreciated that optimum dosage will be determined by standard methods for each treatment modality and indication, taking into account the indication, its severity, route of administration, complicating conditions and the like.

The polynucleotides, polypeptides, agonists and antagonists that are polypeptides of this invention may be employed in accordance with the present invention by expression of such polypeptides *in vivo*, in treatment modalities often referred to as "gene therapy."

Thus, for example, cells from a patient may be engineered with a polynucleotide, such as a DNA or RNA, encoding a polypeptide *ex vivo*, and the engineered cells then can be provided to a patient to be treated with the polypeptide. For example, cells may be engineered *ex vivo* by the use of a retroviral plasmid vector containing RNA encoding a polypeptide of the present invention. Such methods are well-known in the art and their use in the present invention will be apparent from the teachings herein.

Similarly, cells may be engineered *in vivo* for expression of a polypeptide *in vivo* by procedures known in the art. For example, a polynucleotide of the invention may be engineered for expression in a replication defective retroviral vector, as discussed above. The retroviral expression construct then may be isolated and introduced into a packaging cell is transduced with a retroviral plasmid vector containing RNA encoding

a polypeptide of the present invention such that the packaging cell now produces infectious viral particles containing the gene of interest. These producer cells may be administered to a patient for engineering cells *in vivo* and expression of the polypeptide *in vivo*. These and other methods for administering a polypeptide of the present invention by such method should be apparent to those skilled in the art from the teachings of the present invention.

Retroviruses from which the retroviral plasmid vectors herein above mentioned may be derived include, but are not limited to, Moloney Murine Leukemia Virus, spleen necrosis virus, retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, gibbon ape leukemia virus, human immunodeficiency virus, adenovirus, Myeloproliferative Sarcoma Virus, and mammary tumor virus. In one embodiment, the retroviral plasmid vector is derived from Moloney Murine Leukemia Virus.

Such vectors well include one or more promoters for expressing the polypeptide. Suitable promoters which may be employed include, but are not limited to, the retroviral LTR; the SV40 promoter; and the human cytomegalovirus (CMV) promoter described in Miller *et al.*, *Biotechniques*, 7: 980-990 (1989), or any other promoter (*e.g.*, cellular promoters such as eukaryotic cellular promoters including, but not limited to, the histone, RNA polymerase III, and β -actin promoters). Other viral promoters which may be employed include, but are not limited to, adenovirus promoters, thymidine kinase (TK) promoters, and B19 parvovirus promoters. The selection of a suitable promoter will be apparent to those skilled in the art from the teachings contained herein.

The nucleic acid sequence encoding the polypeptide of the present invention will be placed under the control of a suitable promoter. Suitable promoters which may be employed include, but are not limited to, adenoviral promoters, such as the adenoviral major late promoter; or heterologous promoters, such as the cytomegalovirus (CMV) promoter; the respiratory syncytial virus (RSV) promoter; inducible promoters, such as the MMT promoter, the metallothionein promoter; heat shock promoters; the albumin

promoter; the ApoA1 promoter; human globin promoters; viral thymidine kinase promoters, such as the Herpes Simplex thymidine kinase promoter; retroviral LTRs (including the modified retroviral LTRs herein above described); the β -actin promoter; and human growth hormone promoters. The promoter also may be the native promoter which controls the gene encoding the polypeptide.

The retroviral plasmid vector is employed to transduce packaging cell lines to form producer cell lines. Examples of packaging cells which may be transfected include, but are not limited to, the PE501, PA317, Y-2, Y-AM, PA12, T19-14X, VT-19-17-H2, YCRE, YCRIP, GP+E-86, GP+envAm12, and DAN cell lines as described in Miller, A., *Human Gene Therapy*, 1: 5-14 (1990). The vector may be transduced into the packaging cells through any means known in the art. Such means include, but are not limited to, electroporation, the use of liposomes, and CaPO_4 precipitation. In one alternative, the retroviral plasmid vector may be encapsulated into a liposome, or coupled to a lipid, and then administered to a host.

The producer cell line will generate infectious retroviral vector particles, which include the nucleic acid sequence(s) encoding the polypeptides. Such retroviral vector particles then may be employed to transduce eukaryotic cells, either in vitro or in vivo. The transduced eukaryotic cells will express the nucleic acid sequence(s) encoding the polypeptide. Eukaryotic cells which may be transduced include, but are not limited to, embryonic stem cells, embryonic carcinoma cells, as well as hematopoietic stem cells, hepatocytes, fibroblasts, myoblasts, keratinocytes, endothelial cells, and bronchial epithelial cells.

The present invention is further described by the following examples. The examples are provided solely to illustrate the invention by reference to specific embodiments. These exemplification's, while illustrating certain specific aspects of the invention, do not portray the limitations or circumscribe the scope of the disclosed invention.

All examples were carried out using standard techniques, which are well known and routine to those of skill in the art, except where otherwise described in detail. Routine molecular biology techniques of the following examples can be carried out as described in standard laboratory manuals, such as Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), herein referred to as "Sambrook."

All parts or amounts set out in the following examples are by weight, unless otherwise specified. Unless otherwise stated size separation of fragments in the examples below was carried out using standard techniques of agarose and polyacrylamide gel electrophoresis ("PAGE") in Sambrook and numerous other references such as, for instance, by Goeddel *et al.*, *Nucleic Acids Res.*, 8: 4057 (1980). Unless described otherwise, ligations were accomplished using standard buffers, incubation temperatures and times. approximately equimolar amounts of the DNA fragments to be ligated and approximately 10 units of T4 DNA ligase ("ligase") per 0.5 μ g of DNA.

Example 1

Expression and Purification of Soluble Form of Human TNF Delta and TNF Epsilon Using Bacteria

The DNA sequence encoding human TNF delta or TNF epsilon in the deposited polynucleotide was amplified using PCR oligonucleotide primers specific to the amino acid carboxyl terminal sequence of the human TNF delta or TNF epsilon protein and to vector sequences 3' to the gene. Additional nucleotides containing restriction sites to facilitate cloning were added to the 5' and 3' sequences respectively.

The 5' oligonucleotide primer had the sequence 5' GCG GGA TCC CAG AGC CTC ACC ACA G 3' containing the underlined restriction site, followed by 16

nucleotides of coding sequence set out in the Figures beginning with the 115th base of the ATG codon.

The 3' primer has the sequence 5' CGC AAG CTT ACA ATC ACA GTT TCA CAA AC 3' contains the underlined HindIII restriction site followed by 20 nucleotides complementary to the last 13 nucleotides of the coding sequence set out in Figures 1 and 2, including the stop codon.

The restrictions sites were convenient to restriction enzyme sites in the bacterial expression vectors pQE-9, which were used for bacterial expression in these examples. (Qiagen, Inc. Chatsworth, CA). pQE-9 encodes ampicillin antibiotic resistance ("Amp^r") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), a 6-His tag and restriction enzyme sites.

The amplified human TNF delta DNA and the vector pQE-9 both were digested with BamHI and HindIII and the digested DNAs then were ligated together. Insertion of the TNF delta DNA into the pQE-9 restricted vector placed the TNF delta coding region downstream of and operably linked to the vector's IPTG-inducible promoter and in-frame with an initiating AUG appropriately positioned for translation of TNF delta.

The ligation mixture was transformed into competent *E. coli* cells using standard procedures. Such procedures are described in Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, 2nd Ed.: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989). *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses lac repressor and confers kanamycin resistance ("Kan^r"), was used in carrying out the illustrative example described here. This strain, which is only one of many that are suitable for expressing TNF delta, is available commercially from Qiagen. Transformants were identified by their ability to grow on LB plates in the presence of ampicillin. Plasmid DNA was isolated from resistant colonies and the identity of the cloned DNA was confirmed by restriction analysis.

Clones containing the desired constructs were grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 ug/ml) and kanamycin (25 µg/ml). The O/N culture was used to inoculate a large culture, at a dilution of approximately 1:100 to 1:250. The cells were grown to an optical density at 600nm ("OD₆₀₀") of between 0.4 and 0.6. Isopropyl-B-D-thiogalactopyranoside ("IPTG") was then added to a final concentration of 1 mM to induce transcription from lac repressor sensitive promoters, by inactivating the lacI repressor. Cells subsequently were incubated further for 3 to 4 hours. Cells then were harvested by centrifugation and disrupted, by standard methods. Inclusion bodies were purified from the disrupted cells using routine collection techniques, and protein was solubilized from the inclusion bodies into 8M urea. The 8M urea solution containing the solubilized protein was passed over a PD-10 column in 2X phosphate buffered saline ("PBS"), thereby removing the urea, exchanging the buffer and refolding the protein. The protein was purified by a further step of chromatography to remove endotoxin. Then, it was sterile filtered. The sterile filtered protein preparation was stored in 2X PBS at a concentration of 95 micrograms per mL.

Analysis of the preparation of TNF delta by standard methods of polyacrylamide gel electrophoresis revealed that the preparation contained about 80% monomer having the expected molecular weight of, approximately, 20.8 kDa.

The protein is purified by chromatography on a nickel-chelate column under conditions that allow for type-binding by proteins containing the 6-HIS tag. The protein is eluted from the column in 6-molar guanidine HCl pH 5.0 and renatured.

Example 2

Cloning and Expression of Soluble Human TNF Delta and TNF Epsilon in a Baculovirus Expression System

The cDNA sequence encoding the full length human TNF delta or TNF epsilon protein, in the deposited clone is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the gene:

The 5' primer has the sequence 5' GCG GGA TCC CCA GAG CCT CAC CAC AG 3' containing the underlined BamHI restriction enzyme site followed by 16 bases of the sequence of TNF delta or TNF epsilon of Figures 1 and 2. Inserted into an expression vector, as described below, the 5' end of the amplified fragment encoding human TNF delta or TNF epsilon provides an efficient signal peptide. An efficient signal for initiation of translation in eukaryotic cells, as described by Kozak, M., J. Mol. Biol. 196: 947-950 (1987) is appropriately located in the vector portion of the construct.

The 3' primer has the sequence 5' CGC TCT AGA ACA ATC ACA GTT TCA CAA AC 3' containing the underlined XbaI restriction site followed by nucleotides complementary to the last 13 nucleotides of the TNF delta or TNF epsilon coding sequence set out in Figures 1 and 2, including the stop codon.

The amplified fragment is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with BamHI and Asp718 and again is purified on a 1% agarose gel. This fragment is designated herein F2.

The vector pA2GP is used to express the TNF delta or TNF epsilon protein in the baculovirus expression system. using standard methods, such as those described in Summers et al, A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experimental Station Bulletin No. 1555 (1987). This expression vector contains the strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcMNPV) followed by convenient restriction sites. The signal peptide of AcMNPV gp67, including the N-terminal methionine, is located just

upstream of a BamHI site. The polyadenylation site of the simian virus 40 ("SV40") is used for efficient polyadenylation. For an easy selection of recombinant virus the beta-galactosidase gene from *E.coli* is inserted in the same orientation as the polyhedrin promoter and is followed by the polyadenylation signal of the polyhedrin gene. The polyhedrin sequences are flanked at both sides by viral sequences for cell-mediated homologous recombination with wild-type viral DNA to generate viable virus that express the cloned polynucleotide.

Many other baculovirus vectors could be used in place of pA2-GP, such as pAc373, pVL941 and pAcIM1 provided, as those of skill readily will appreciate, that construction provides appropriately located signals for transcription, translation, trafficking and the like, such as an in-frame AUG and a signal peptide, as required. Such vectors are described in Luckow *et al.*, *Virology*, 170:31-39, among others.

The plasmid is digested with the restriction enzymes BamHI and XbaI and then is dephosphorylated using calf intestinal phosphatase, using routine procedures known in the art. The DNA is then isolated from a 1% agarose gel using a commercially available kit ("GeneClean" BIO 101 Inc., La Jolla, Ca.). This vector DNA is designated herein "V2".

Fragment F2 and the dephosphorylated plasmid V2 are ligated together with T4 DNA ligase. *E.coli* HB101 cells are transformed with ligation mix and spread on culture plates. Bacteria are identified that contain the plasmid with the human TNF delta or TNF epsilon gene by digesting DNA from individual colonies using BamHI and XbaI and then analyzing the digestion product by gel electrophoresis. The sequence of the cloned fragment is confirmed by DNA sequencing. This plasmid is designated herein pBacTNF delta.

5 μ g of the plasmid pBacTNF delta is co-transfected with 1.0 μ g of a commercially available linearized baculovirus DNA ("BaculoGold™ baculovirus DNA",

Pharmingen, San Diego, CA.), using the lipofection method described by Felgner *et al.*, *Proc. Natl. Acad. Sci. USA*, 84:7413-7417 (1987). 1 μ g of BaculoGold™ virus DNA and 5 μ g of the plasmid pBacTNF delta are mixed in a sterile well of a microtiter plate containing 50 μ l of serum free Grace's medium (Life Technologies Inc., Gaithersburg, MD). Afterwards 10 μ l Lipofectin plus 90 μ l Grace's medium are added, mixed and incubated for 15 minutes at room temperature. Then the transfection mixture is added drop-wise to Sf9 insect cells (ATCC CRL 1711) seeded in a 35 mm tissue culture plate with 1 ml Grace's medium without serum. The plate is rocked back and forth to mix the newly added solution. The plate is then incubated for 5 hours at 27°C. After 5 hours the transfection solution is removed from the plate and 1 ml of Grace's insect medium supplemented with 10% fetal calf serum is added. The plate is put back into an incubator and cultivation is continued at 27°C for four days.

After four days the supernatant is collected and a plaque assay is performed, as described by Summers and Smith, cited above. An agarose gel with "Blue Gal" (Life Technologies Inc., Gaithersburg) is used to allow easy identification and isolation of gal-expressing clones, which produce blue-stained plaques. (A detailed description of a "plaque assay" of this type can also be found in the user's guide for insect cell culture and baculovirology distributed by Life Technologies Inc., Gaithersburg, page 9-10).

Four days after serial dilution, the virus is added to the cells. After appropriate incubation, blue stained plaques are picked with the tip of an Eppendorf pipette. The agar containing the recombinant viruses is then resuspended in an Eppendorf tube containing 200 μ l of Grace's medium. The agar is removed by a brief centrifugation and the supernatant containing the recombinant baculovirus is used to infect Sf9 cells seeded in 35 mm dishes. Four days later the supernatants of these culture dishes are harvested and then they are stored at 4°C. A clone containing properly inserted TNF delta or TNF epsilon is identified by DNA or TNF epsilon analysis including restriction mapping and sequencing. This is designated herein as V-TNF delta.

Sf9 cells are grown in Grace's medium supplemented with 10% heat-inactivated FBS. The cells are infected with the recombinant baculovirus V-TNF delta at a multiplicity of infection ("MOI") of about 2 (about 1 to about 3). Six hours later the medium is removed and is replaced with SF900 II medium minus methionine and cysteine (available from Life Technologies Inc., Gaithersburg). 42 hours later, 5 μ Ci of 35S-methionine and 5 μ Ci 35S cysteine (available from Amersham) are added. The cells are further incubated for 16 hours and then they are harvested by centrifugation, lysed and the labeled proteins are visualized by SDS-PAGE and autoradiography.

Example 3

Tissue Distribution of TNF Delta Expression

Northern blot analysis was carried out to examine the levels of expression of TNF delta in human tissues, using methods described by, among others, Sambrook *et al.*, cited above. Total cellular RNA samples are isolated with RNazol™ B system (Biotech Laboratories, Inc. 6023 South Loop East, Houston, TX 77033).

About 10 μ g of Total RNA was isolated from tissue samples. The RNA was size resolved by electrophoresis through a 1% agarose gel under strongly denaturing conditions. RNA was blotted from the gel onto a nylon filter, and the filter then is prepared for hybridization to a detectably labeled polynucleotide probe.

As a probe to detect mRNA that encodes TNF delta, the antisense strand of the coding region of the cDNA insert in the deposited clone was labeled to a high specific activity. The cDNA was labeled by primer extension, using the Prime-It kit, available from Stratagene. The reaction was carried out using 50 ng of the cDNA, following the standard reaction protocol as recommended by the supplier. The labeled polynucleotide was purified away from other labeled reaction components by column chromatography using a Select-G-50 column, obtained from 5-Prime - 3-Prime, Inc. of 5603 Arapahoe Road, Boulder, CO 80303.

The labeled probe was hybridized to the filter, at a concentration of 1,000,000 cpm/ml, in a small volume of 7% SDS, 0.5 M NaPO₄, pH 7.4 at 65°C, overnight.

Thereafter the probe solution was drained and the filter is washed twice at room temperature and twice at 60°C with 0.5 x SSC, 0.1% SDS. The filter then is dried and exposed to film at -70°C overnight with an intensifying screen.

Autoradiography shows that mRNA for TNF delta was detected in all 16 tissues with highest expression in heart followed by placenta and kidney.

Example 4

Gene Therapeutic Expression of Human TNF Delta or TNF Epsilon

Fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature overnight. After 24 hours at room temperature, the flask is inverted - the chunks of tissue remain fixed to the bottom of the flask - and fresh media is added (*e.g.*, Ham's F12 media, with 10% FBS, penicillin and streptomycin). The tissue is then incubated at 37°C for approximately one week. At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerges. The monolayer is trypsinized and scaled into larger flasks.

A vector for gene therapy is digested with restriction enzymes for cloning a fragment to be expressed. The digested vector is treated with calf intestinal phosphatase to prevent self-ligation. The dephosphorylated, linear vector is fractionated on an agarose gel and purified.

cDNA capable of expressing active TNF delta or TNF epsilon, is isolated. The ends of the fragment are modified, if necessary, for cloning into the vector. For instance, 5' overhanging may be treated with DNA polymerase to create blunt ends. 3' overhanging ends may be removed using S1 nuclease. Linkers may be ligated to blunt ends with T4 DNA ligase.

Equal quantities of the Moloney murine leukemia virus linear backbone and the TNF delta or TNF epsilon fragment are mixed together and joined using T4 DNA ligase. The ligation mixture is used to transform E. Coli and the bacteria are then plated onto agar-containing kanamycin. Kanamycin phenotype and restriction analysis confirm that the vector has the properly inserted gene.

Packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagle's Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The vector containing the TNF delta or TNF epsilon gene is introduced into the packaging cells by standard techniques. Infectious viral particles containing the TNF delta or TNF epsilon gene are collected from the packaging cells, which now are called producer cells.

Fresh media is added to the producer cells, and after an appropriate incubation period media is harvested from the plates of confluent producer cells. The media, containing the infectious viral particles, is filtered through a Millipore filter to remove detached producer cells. The filtered media then is used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the filtered media. Polybrene (Aldrich) may be included in the media to facilitate transduction. After appropriate incubation, the media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his, to select out transduced cells for expansion.

Engineered fibroblasts then may be injected into rats, either alone or after having been grown to confluence on microcarrier beads, such as cytodex 3 beads. The injected fibroblasts produce TNF delta or TNF epsilon product, and the biological actions of the protein are conveyed to the host.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

WHAT IS CLAIMED IS:

1. An isolated polynucleotide comprising a polynucleotide having at least 70% identify to a member selected from the group consisting of:

(a) a polynucleotide encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:2;

(b) a polynucleotide encoding a polypeptide comprising an amino acid sequence as set forth in SEQ ID NO:4;

(c) a polynucleotide encoding a polypeptide comprising amino acid 39 to amino acid 233 of SEQ ID NO:2;

(d) a polynucleotide which is complementary to the polynucleotide of (a), (b), or (c); and

(e) a polynucleotide comprising at least 15 bases of the polynucleotide of (a), (b), (c) or (d).

2. The polynucleotide of Claim 1 wherein the polynucleotide is DNA.

3. The polynucleotide of Claim 1 wherein the polynucleotide is RNA.

4. The polynucleotide of Claim 1 wherein the polynucleotide is genomic DNA.

5. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids of SEQ ID NO:2.

6. The polynucleotide of Claim 2 which encodes a polypeptide comprising amino acid 39 to 233 of SEQ ID NO:2.

7. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids of SEQ ID NO:4.

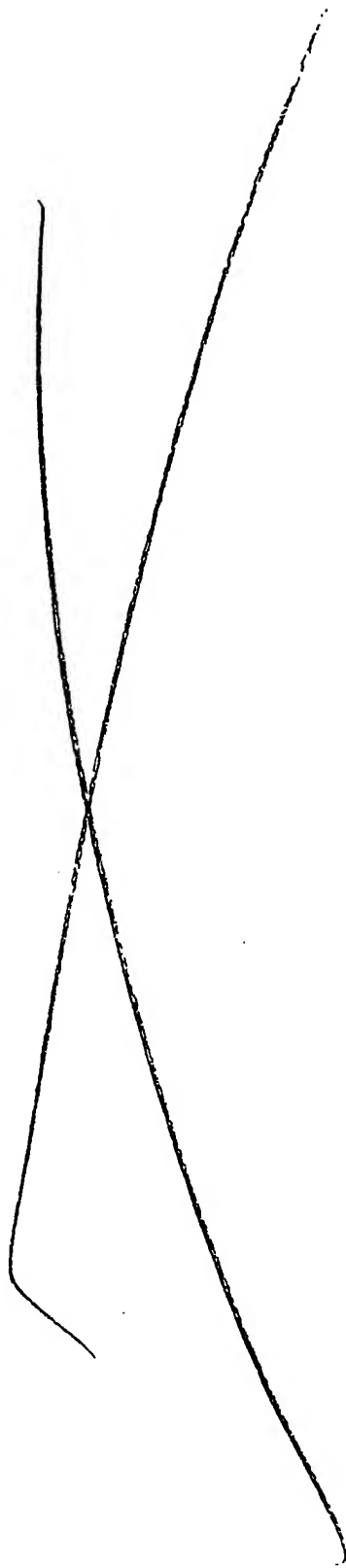
8. The polynucleotide of Claim 2 which encodes a polypeptide comprising the amino acids 1 to 188 of SEQ ID NO:4.
9. An isolated polynucleotide comprising a polynucleotide which is at least 70% identical to a member selected from the group consisting of:
 - (a) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the human cDNA contained in ATCC Deposit No. 97377;
 - (b) a polynucleotide which encodes a mature polypeptide having the amino acid sequence expressed by the human cDNA contained in ATCC Deposit No. 97457;
 - (c) a polynucleotide which is complementary to the polynucleotide of (a) or (b); and
 - (d) a polynucleotide comprising at least 15 bases of the polynucleotide of (a), (b) or (c).
10. The polynucleotide of claim 1 comprising from nucleotide 447 to nucleotide 1717 of SEQ ID No. 1.
11. The polynucleotide of Claim 1 comprising from nucleotide 332 to nucleotide 1717 of SEQ ID No. 1.
12. The polynucleotide of Claim 1 comprising from nucleotide 1 to nucleotide 564 of SEQ ID NO:3.
13. A vector comprising the DNA of Claim 2.
14. A host cell comprising the vector of Claim 13.

15. A process for producing a polypeptide comprising expressing from the host cell of Claim 14 the polypeptide encoded by said DNA.
16. A process for producing a cell comprising genetically engineering the cell with the vector of Claim 12 to thereby express the polypeptide encoded by the human cDNA contained in the vector.
17. A polypeptide comprising a member selected from the group consisting of:
 - (a) a polypeptide having an amino acid sequence as set forth in SEQ ID NO:2; and
 - (b) a polypeptide comprising amino acid 39 to 233 of SEQ ID NO:2;
 - (c) a polypeptide comprising amino acid 1 to 188 of SEQ ID NO:4; and
 - (d) a polypeptide having at least a 70% identity to the polypeptide of (a), (b) or (c).
18. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 1 to amino acid 233 of SEQ ID NO:2.
19. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 39 to amino acid 233 of SEQ ID NO:2.
20. The polypeptide of Claim 17 wherein the polypeptide comprises amino acid 1 to amino acid 188 of SEQ ID NO:4.
21. A compound which inhibits activation of the polypeptide of claim 17.
22. A method for the treatment of a patient having need of TNF delta comprising administering to the patient a therapeutically effective amount of the polypeptide of claim 17.

23. A method for the treatment of a patient having need of TNF epsilon comprising: administering to the patient a therapeutically effective amount of the polypeptide of claim 17.
24. The method of Claim 22 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
25. The method of Claim 23 wherein said therapeutically effective amount of the polypeptide is administered by providing to the patient DNA encoding said polypeptide and expressing said polypeptide *in vivo*.
26. A method for the treatment of a patient having need to inhibit a TNF delta polypeptide comprising: administering to the patient a therapeutically effective amount of the compound of Claim 21.
27. A method for the treatment of a patient having need to inhibit a TNF epsilon polypeptide comprising administering to the patient a therapeutically effective amount of the compound of Claim 21.
28. A process for diagnosing a disease or a susceptibility to a disease related to an under-expression of the polypeptide of claim 17 comprising determining a mutation in a nucleic acid sequence encoding said polypeptide.
29. A diagnostic process comprising analyzing for the presence of the polypeptide of claim 17 in a sample derived from a host.
30. A method for identifying compounds which bind to and inhibit activation of the polypeptide of claim 17 comprising

contacting a cell expressing on the surface thereof a receptor for the polypeptide, said receptor being associated with a second component capable of providing a detectable signal in response to the binding of a compound to said receptor, with an analytically detectable TNF delta polypeptide and a compound under conditions to permit binding to the receptor; and

determining whether the compound binds to and inhibits the receptor by detecting the absence of a signal generated from the interaction of the TNF delta with the receptor.

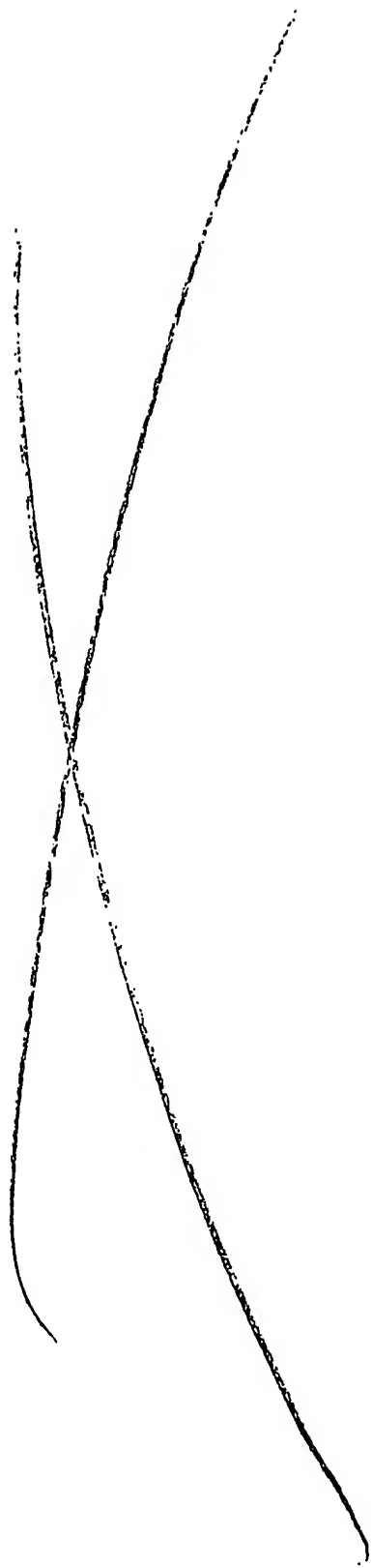


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FIG. 1A

1 ACCTCTGTCTTAGAGGGACTGGAACCTAATTCTCTGAGCCTGAGGGAGGGTGGAGGG 60
 61 TCTCAAGACAACGCTGTCCCCACGAGCGAGTGCCAGGAGCACTAACAGTACCCCTTAGATT 120
 121 GCTTTCCTCCTCCCTCTTTTATTTTCAAGTCTCTTTTATTTCTCTCTGCGTAACAA 180
 181 CCTTCTTCCCTTCTGCAACCACTGCCCGTACCCTTACCCGCGCCACCTCCTTGTCTACA 240
 241 CCACTCTTGAACCAACAGCTGTGTCAGGGTCCCCAGCTCATGCCAGCCTCATCTCCTT 300
 301 TCTTGCTAGCCCCCAAGGGCCTCCAGGCAACATGGGGGGCCCAAGTCAGAGAGCCGGCAC 360
 M G G P V R E P A L
 361 TCTCAGTTGCCCTCTGTGTTAGTTGGGGGGCAGCTCTGGGGGGCCGTGGCTTGTGCCATGG 420
 S V A L W L S W G A A L G A V A C A M A
 421 CTCTGCTGACCCCAACAACAGAGCTGCAGAGCCTCAGGAGAGAGGTGAGCCGGCTGCAGA 480
 L L T Q Q T E L Q S L R R E V S R L Q R
 481 GGACAGAGGGCCCTCCAGAAATGGGGAAGGTATCCCTGCAGAGTCTCCCGGAGCAGA 540
 T G G P S Q N G E G Y P W Q S L P E Q S
 541 GTTCCGATGCCCTGGAAGCCTGGGAGAAATGGGGAGAGATCCCGGAAAGGAGAGCAGTGC 600
 S D A L E A W E N G E R S R K R R A V L
 601 TCAGCCAAACAGAGAAGCAGCACTCTGTCTCTGCACCTGGTTCCCATTAACGCCACCT 660
 T Q K Q K K Q H S V L H L V P I N A T S
 661 CCAAGGATGACTCCGATGTGACAGAGGTGATGTGGCAACCAGCTCTTAGCGGTGGAGAG 720
 K D D S D V T E V M W Q P A L R R G R G

MATCH WITH FIG. 1B



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FIG. 1B

MATCH WITH FIG. 1A

721	GCCTACAGGCCCAAGCATATGGTGTCCGAATCCAGGATGCTGAGTTTATCTGCTGTATA	780
	L Q A Q G Y G V R I Q D A G V Y L L Y S	
781	GCCAGGTCCTGTTTCAAGACGTGACTTTCACCATGGGTCAGGTGGTGTCTCGAAGGCC	840
	Q V L F Q D V T F T M G Q V V S R E G Q	
841	AAGGAAGCAGGAGACTCTATTCCGATGTATAAGAGTATGCCCTCCACCCGACCGCG	900
	G R Q E T L F R C I R S M P S H P D R A	
901	CCTACAACAGCTGCTATAGCGCAGGTGCTCTTCCATTACACCAAGGGATATTCTGAGTG	960
	Y N S C Y S A G V F H L H Q G D I L S V	
961	TCATAATTCCCGCGCAAGCGCGAACTTAACCTCTCTCCACATGGAACCTTCTCTGGGT	1020
	I I P P A R A F L N L S P H G T F L G F	
1021	TTGTGAAACTGTGATTGTGTATAAAAGTGGCTCCAGCTTGGAAAGACCAGGGTGGGTA	1080
1081	CATACTGGAGACAGCCAGAGCTCAGTATATAAAGGAGAGGGAATGTGCAGGAACAGAGG	1140
1141	CGTCTTCTGGGTTTGGCTCCCCGTTCTCTCACTTTTCCCTTTTCAATCCACCCCTAGA	1200
1201	CTTTGATTTTACGGATATCTTCTCTTCTTCCCATGAGCTCCGAATCTTGGGTGTGT	1260
1261	GATGATGAGGGCGCGGACGCCGCCAGCCATTCTCCAGACCTGGTGGGGCCCACTGG	1320
1261	AAGCATCCAGAAACAGACCAACCATCTACCGCGCGCTCTAGAGGATCCCTCGAGGGGCCCA	1380
1381	AGCTTACGCGTCCATCGACGTCATAGCTCTCTCTCCCTATAGTGAGTCGTATTATAAGCTA	1440
1441	GCTTGGGATCTTTCTGAAGGAACCTTACTTCTGTGTGTGACATAATTGGACAACTACC	1500
1501	TACAGAGATTTAAAGCTCTAAGCTAAATATAAAATTTTAAAGTGTATAATGTGTAAACT	1560
1561	AGCTGCATATGCTTCTGCTTCAGAGCTTTGGCTTACTGAGTATGATATGAATAATATAT	1620
1621	ACACAGGAGCTAGTCATCTATGTTGCTTTTAGATCAAGCCAAAGGTCAATTCAGGCCTCAGC	1680
1681	TCAAGCTGTATCATATATCAGCATACAAATTGTGAG	1717

X

FIG. 2A

GGGACAGGAGGCCCTCCAGAAATGGGAAGGTATCCCTGGCAGAGTCTCCGGAGCA
G T G G P S Q N G E G Y P W Q S L P E Q

GAGTCCGATGCCCTGGAAGCCTGGGAGAGTGGGAGAGATCCCGGAAAGGAGAGCAGT
S S D A L E A W E S G E R S R K R R A V

GCTACCCAAACAGAAATGACTCCGATGTGACAGAGGTGATGTGGCAACCAGCTCT
L T Q K Q K N D S D V T E V M W Q P A L

TAGCGTGGAGAGGCCCTACAGGCCCAAGGATATGGTGTCGGAATCCAGGATGCTGGAGT
R R G R G L Q A Q G Y G V R I Q D A G V

TTATCTCCTGTATAGCCAGGTCCTGTTCAGACGTGACTTTCACCATGGGTCAGGTGGT
Y L L Y S Q V L F Q D V T F T M G Q V V

GTCTCGAAGGCCAAGGAAGCAGGAGACTCTATTCGGATGTATAAGAAGTATGCCCTC
S R E G Q G R Q E T L F R C I R S M P S

CCACCCGACCGGCTACAACAGCTGCTATAGCGCAGGTGCTTCCATTACACCAAGG
H P D R A Y N S C Y S A G V F H L H Q G

MATCH WITH FIG. 2B

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X

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FIG. 2B

MATCH WITH FIG. 2A

GGATATTCTGAGTGCATAATTCCCCGGCAAGGCGAACTTAACCTCTCTCCACATGG
 D I L S V I I P R A R A K L N L S P H G

AACCTTCCCTGGGTTTGTGAACCTGTGATTGTGTTATAAAAGTGGCTCCAGCTTGGAA

T F L G F V K L

GACCAGGGTGGGTACATACTGGAGACAGCCAAAGAGCTGAGTATATAAGGAGAGGGAATG
 TGCAGGAACAGAGCGTCTTCCCTGGGTTTGGCTCCCCGTTCCCTCACTTTTCCCTTTTCAT
 TCCACCCCCCTAGACTTTTGGATTTTACGGATATCTTGTCTGTCTCCCATGGAGCTCCG
 AATTCTTGCGTGTGTAGATGAGGGCGGGGACGGCGCCAGGCATTGTTTCAGACCTG
 GTCGGGCCCACTGGAAGCATCCAGAACAGCACCACTAGCGCGCTCGAGGGAAGC
 ACCGCGGTTGGCCGAAGTCCACGAAGCCGCTCTGCTAGGGAACCTGGTTCTCCAT
 GCCACAACCTCTCCAGGTGGCTCTGCTCTTCAACCCCAAGAGCCTTAACCTA
 CGTCCCTCTCTCCATCTATCGGACCCAGTTTCCATCACTATCTCCAGAGATGTAGCTAT
 TATGCGCCCGTCTACAGGGGTGCCCCGACGATGACGGTGCCTTCGCAGTCAAAATTACTCT
 TCGGGTCCCAAGGTTTGGCTTTTACGGCTCCATTGCCCGCGGTGGCAGGCCATTCCAA
 GCCCTTCCGGGTGGAACGTGTGTCGGAGGACCTCGGGTGTATCGTACGCCCTGGTGT
 GGTGTTGCCCTCACTCCTCTGAGCTCTTCTTCTGATCAAGCCCTGCTTAAAGTTAAATAA
 AATAGATGAATGATAAAATAAAATAAAATAAAATAAAATAAAATAAAATAAAATAAA

X

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FIG. 3A

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1  MSTESMIRDVELAEALPKKTGGPOGSRRC-LFLSLFSE
1  MTPPERLF-----LPRVCGTT-----LHLILGL
1  MGGFVREPALSV--ALWLSWGAALGAVACAMALLTQOT
1  G-----
70  ISPLAQAV-----RSSSRT-----PSDK--PVAHVVA
41  TPSAACTA-----RQHPKMHLAHSTLK--PAHLIG
67  PEQSSDALEAWENGERSRKRRRAVLTKQKQKQHSVHLMP
18  PEQSSDALEAWESGERSRKRRRAVLTKQKQK-----
118 ELRDNQLVVPSEGLYLIYSQVLFKGQGC----PSTHVLL
93  SLSNNSLLVPTSGIYFVYSQVVFSGKAYSPKAPSSPLYL
137 GVR-----IQDAGVYLLYSQVLFQDVTFT-----M
72  GVR-----IQDAGVYLLYSQVLFQDVTFT-----M
184 GAEAKPWYEPIYLGGVFQLEKGDRLSAEINRPDYLDFAE
158 -GLQEPWLHSMYHGAAFOLTQGDQLSTHTDGIPHVLVS-
186 HPDRA--YNSCYSAQVFIHQGDILSVIIPRARAKLNLS
121 HPDRA--YNSCYSAQVFIHQGDILSVIIPRARAKLNLS

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Decoration '': Shade (with solid black)
residues that match TNFalpha exactly.

Match with FIG. 3B

X

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FIG. 3B

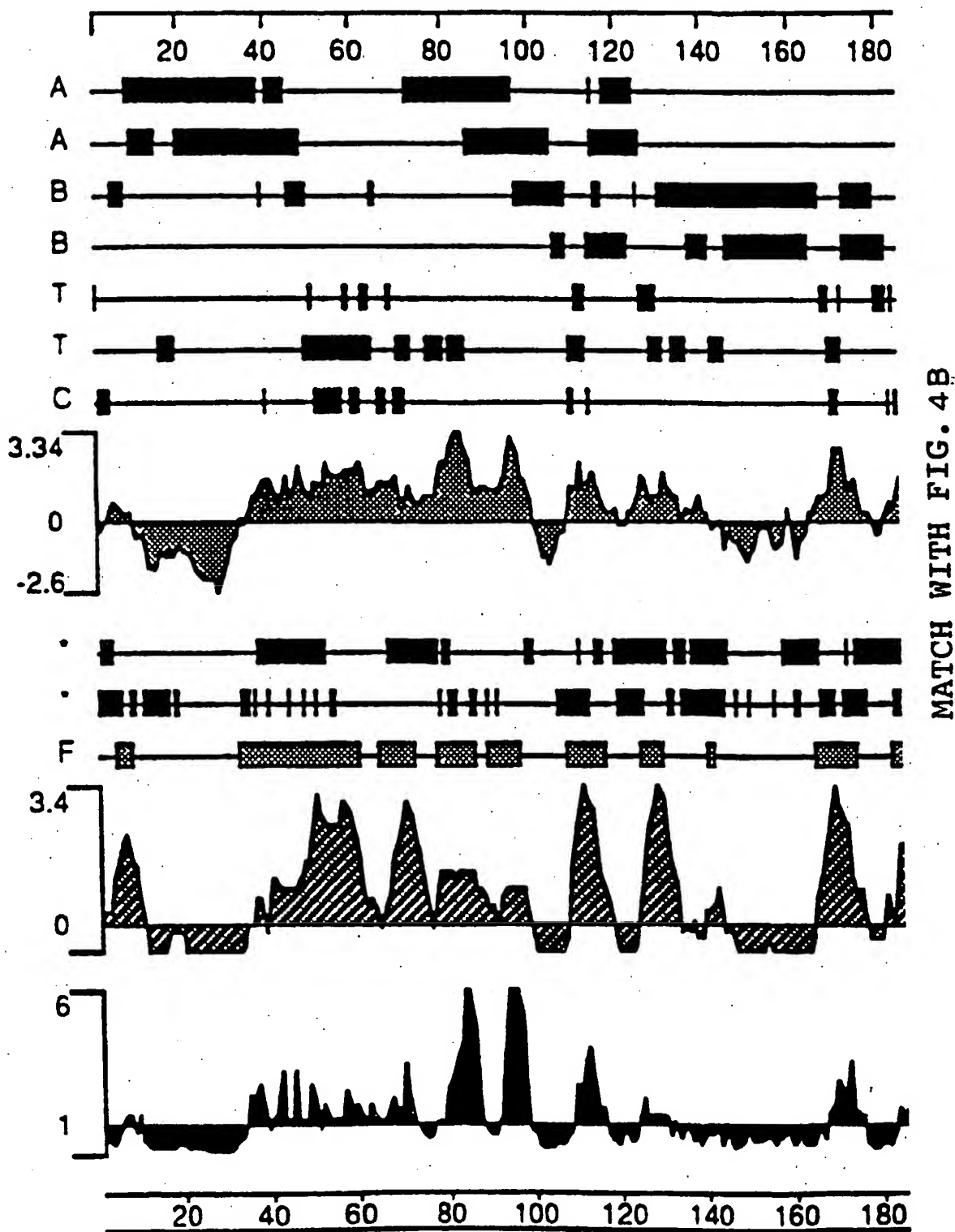
LIVAGATTLFCLLHFGVIGPOREESPRDLSL	TNFalpha
LLV-----LI-----PGAQGLF-GVGL	TNFbeta
ELQSLRREVSRLQRTIGGPSQNGEGYPWQ-SL	TNFdelta
-----TGGPSQNGEGYPWQ-SL	TNFepsilon
-----NPOAEGOLWLN--RRANALLANGV	TNFalpha
-----DESKQNSLIWRA--NTDRAFLQDGF	TNFbeta
INATSKDDSDVTEVMWQPALRRGRGLQACGY	TNFdelta
-----NDSDVTEVMWQPALRRGRGLQACGY	TNFepsilon
THTISRIVASYQTKVNLLSAIKSPCORETPE	TNFalpha
AHEVOLFFSSQYPFHMPLLSQKMVYP-----	TNFbeta
GQVVSREGQG--RQETLFR-----CIRSMPS	TNFdelta
GQVVSREGQG--RQETLFR-----CIRSMPS	TNFepsilon
SGQVYFGIIAL	TNFalpha
PSTVFFCAFAL	TNFbeta
PHGTFLGFVK-L	TNFdelta
PHGTFLGFVKL.	TNFepsilon

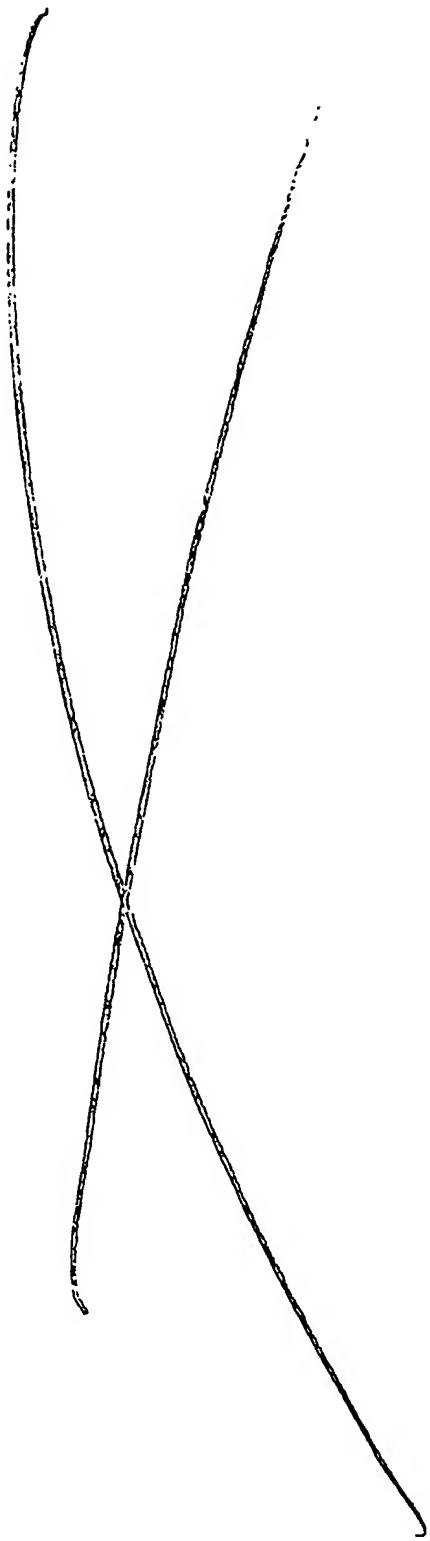
MATCH WITH FIG. 3A

X

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FIG. 4A

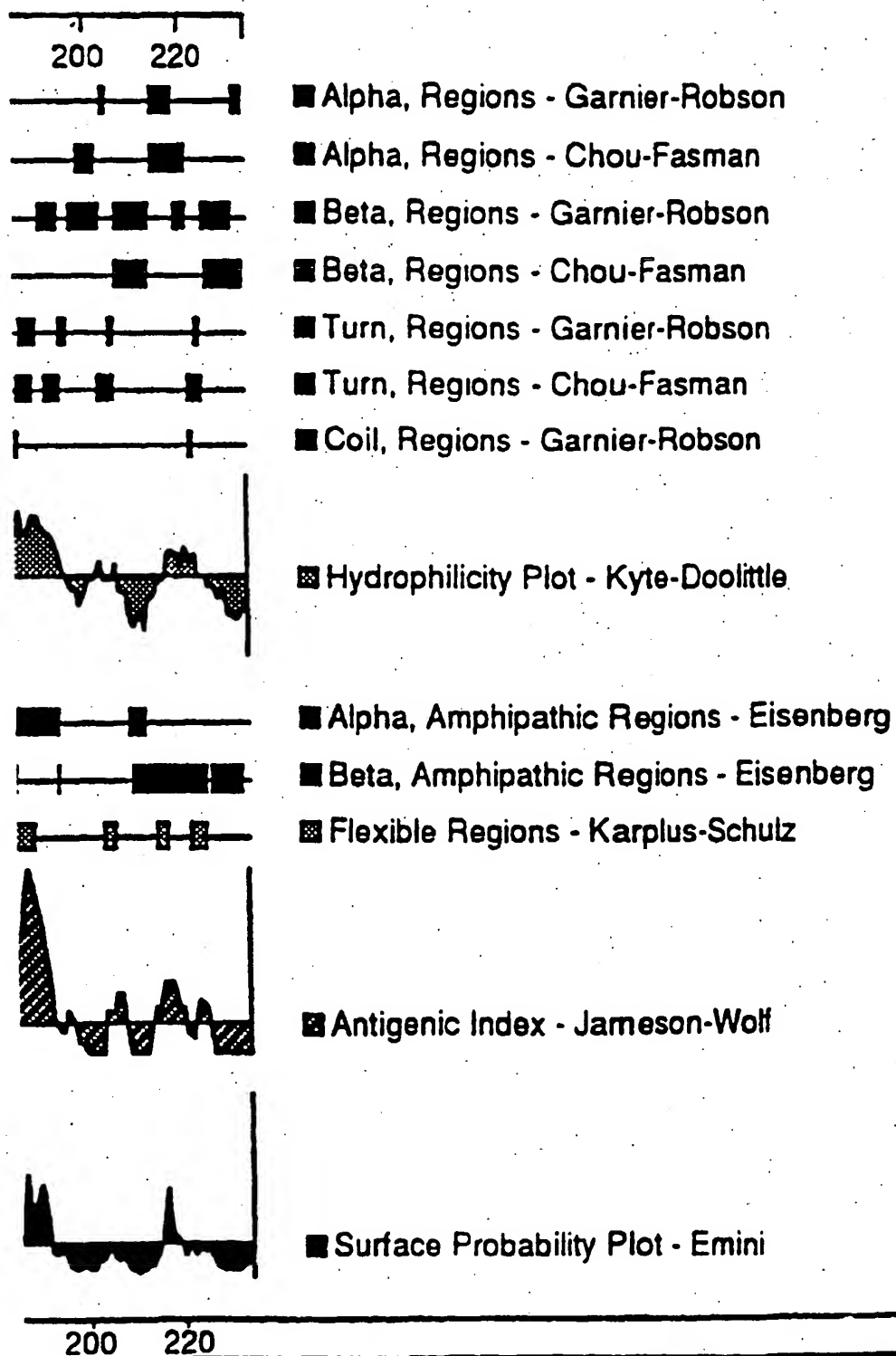


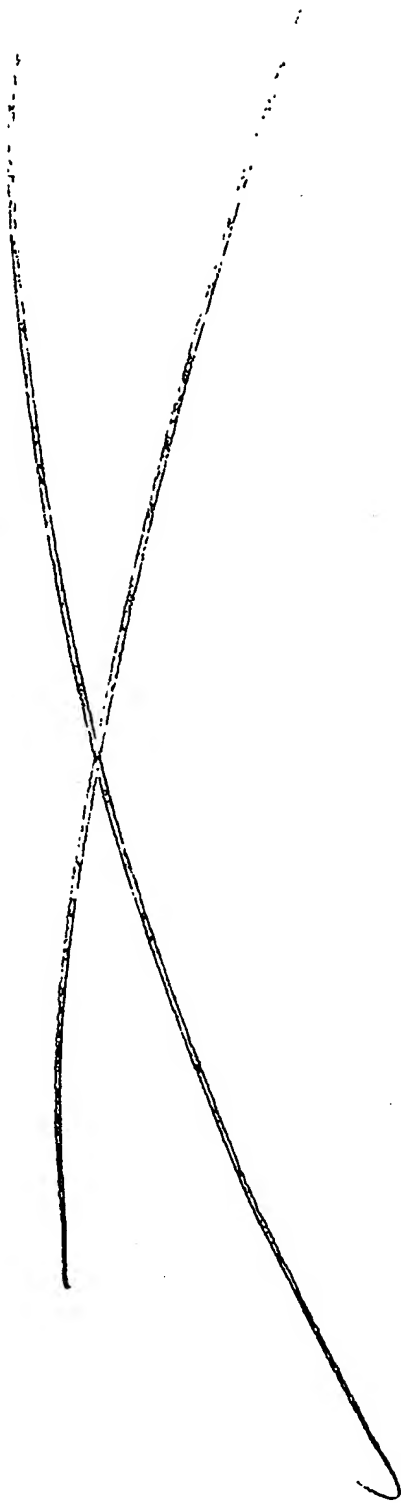


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FIG. 4B

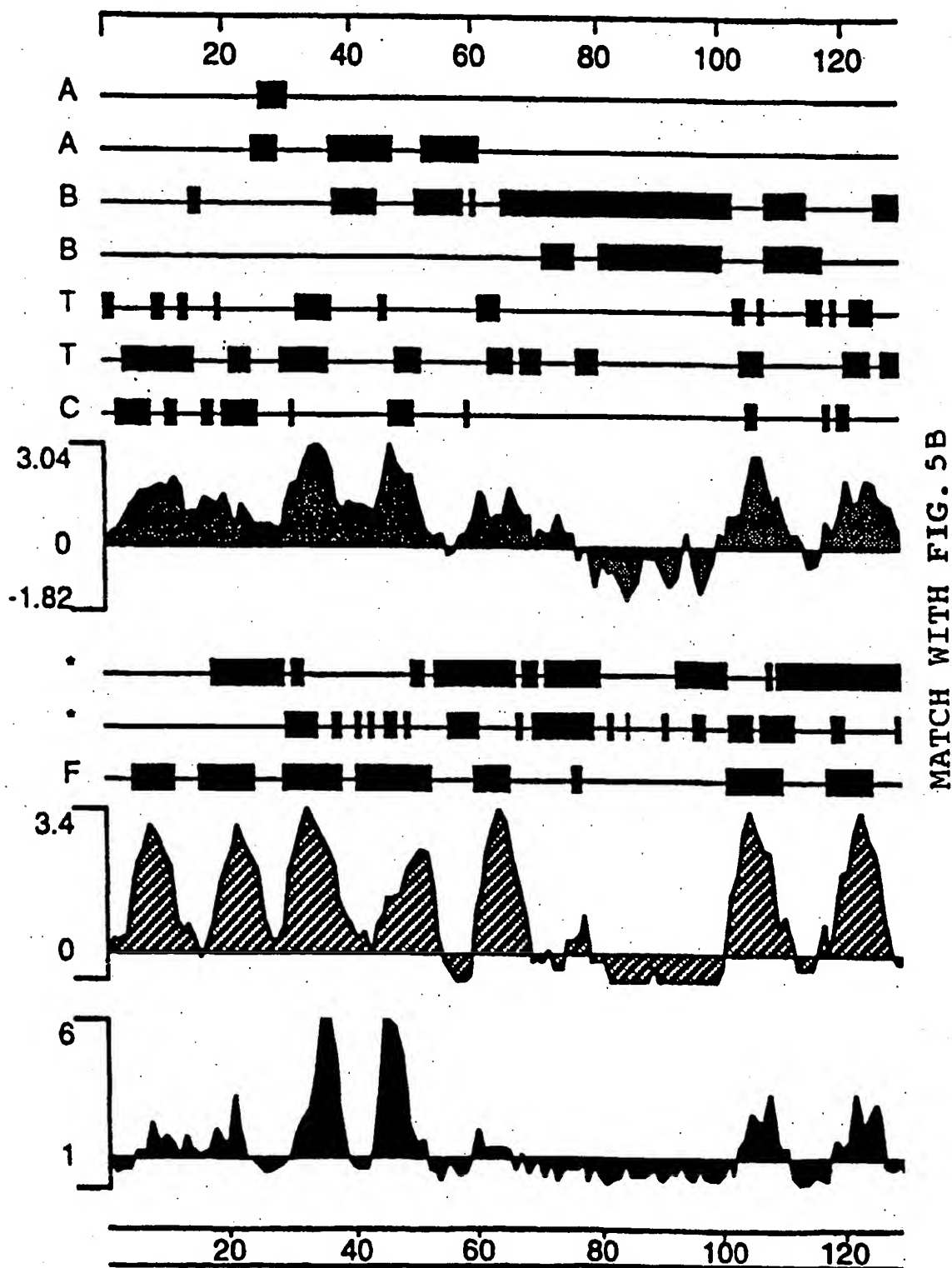
MATCH WITH FIG. 4A





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FIG. 5A

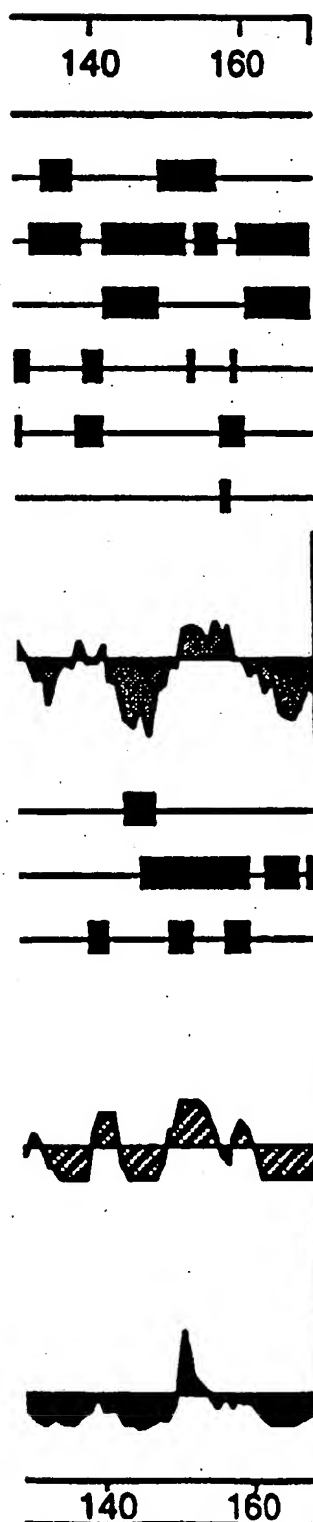


X

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FIG. 5B

MATCH WITH FIG. 5A



■ Alpha, Regions - Garnier-Robson

■ Alpha, Regions - Chou-Fasman

■ Beta, Regions - Garnier-Robson

■ Beta, Regions - Chou-Fasman

■ Turn, Regions - Garnier-Robson

■ Turn, Regions - Chou-Fasman

■ Coil, Regions - Garnier-Robson

■ Hydrophilicity Plot - Kyte-Doolittle

■ Alpha, Amphipathic Regions - Eisenberg

■ Beta, Amphipathic Regions - Eisenberg

■ Flexible Regions - Karplus-Schulz

■ Antigenic Index - Jameson-Wolf

■ Surface Probability Plot - Emini

X

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03774**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(5) : C07H 21/02, 21/04; C07K 1/00, 14/00; C12N 5/00, 15/09, 15/63

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.1, 23.51; 530/350; 435/172.3, 240.1, 252.3, 320.1, 814; 935/1, 3, 23, 60

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN, MEDLINE, BIOSIS, SCISEARCH, DIALOG, DERWENT
search terms: TNF, alpha, beta, delta, epsilon, lymphotoxin, heart**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database on N-Geneseq22, General Hospital Corporation (Edinburgh, UK), No.N90606, SEED, B. 'Rapid immuno:selection cloning-used to clone genes encoding cell surface antigens associated with mammalian T lymphocytes.', disclosure, EP-330191-A, figure 6, August 30, 1989.	1-16
X	Database on EST, Institute for Genome Research, (Gaithersburg, MD), No. M78230, ADAMS, M.D. 'Sequence identification of 2,375 brain genes.' Nature, 355: 632-634, 1992.	1-16
Y	US 5,487,984 (ALLET ET AL) 30 January 1996 (30.01.96), column 2, line 40 to column 3 line 12.	1-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principles or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
I earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 01 JULY 1996	Date of mailing of the international search report 01 AUG/1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DARYL A. BASHAM Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03774

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

1-20

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/03774

A. CLASSIFICATION OF SUBJECT MATTER: US CL

536/23.1, 23.51; 530/350; 435/172.3, 240.1, 252.3, 320.1, 814; 935/1, 3, 23, 60

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

I. Claims 1-20, drawn to an isolated polynucleotide, a vector, a host cell, a process for producing a polypeptide, a process producing a cell and a polypeptide.

II. Claims 21, 26 and 27, drawn to a compound which inhibits activation of the polypeptide and a method for the treatment of a patient comprising administering the compound.

III. Claims 22 and 23, drawn to a method for the treatment of a patient comprising administering a polypeptide.

IV. Claims 24 and 25, drawn to a method for the treatment of a patient comprising administering DNA.

V. Claim 28, drawn to a process for diagnosing a disease comprising determining a mutation.

VI. Claim 29, drawn to a diagnostic process comprising analyzing for a polypeptide.

VII. Claim 30, drawn to a method for identifying compounds.

The DNA and polypeptide compositions of Group I have materially different chemical structures and biological functions from the antagonist of Group II. The special technical features by which the DNA and polypeptide of Group I are defined distinguish them from the special technical features which define the antagonist of Group II. The method of each group I-VII is materially different from the method in any other group because each is practiced with materially different process steps; the process steps are the special technical features which distinguish each method from the others. Because the process steps do not share the same or a corresponding special technical feature, unity of invention is lacking. The claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

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